# TREATMENTS FOR ACUTE FATAL ALPHAVIRAL ENCEPHALOMYELITIS

by

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# ABSTRACT

Inflammation in the nervous system is a necessary part of the response to CNS infection, but can also cause neuronal damage in both infectious and autoimmune diseases of the CNS. Sindbis virus is an enveloped, positive-strand RNA virus that infects neurons and causes acute encephalomyelitis and fatal paralysis in mice. Adult C57BL/6 mice inoculated with the neurovirulent strain of Sindbis virus (NSV) succumb to fatal paralysis despite clearing infectious virus from the central nervous system. The anti-viral immune response rather than viral replication is a major contributor to neuronal damage in NSV infection. This study has evaluated two potential methods for treatment of alphavirus encephalomyelitis. Both methods involve small molecules with neuroprotective properties that also inhibit the peripheral immune response in distinct ways.

DON (6-diazo-5-oxo-l-norleucine) is a glutamine antagonist initially studied as a chemotherapeutic for various cancers. Because glutamine is necessary for the synthesis of glutamate by glutaminase, proliferating primary T-cells are very sensitive to perturbations in glutamine metabolism. Daily intraperitoneal administration of

DON to NSV-infected C57BL/6 mice inhibited the peripheral immune response to NSV, delayed viral clearance, decreased inflammation in the CNS, and protected NSV infected mice from fatal paralysis. However, once treatment was stopped, CNS inflammation appeared, infectious virus was cleared, and mice developed fatal paralysis. Protection was associated with failure to induce an adaptive immune response in the draining cervical lymph nodes and decreased leukocyte infiltration, lower levels of inflammatory cytokines, and delayed viral clearance in the brain. *In vitro* and *in vivo* studies showed that DON inhibited stimulus-induced proliferation of lymphocytes.

GYKI-52466, an AMPA receptor antagonist, was previously shown to protect against NSV-induced fatal paralysis by being both neuroprotective and anti-inflammatory. GYKI belongs to a class of 2,3 benzodiazepines that allosterically inhibits glutamate gated AMPA receptors in the CNS. In this study, we demonstrated that AMPA receptor antagonists directly affect lymphocyte proliferation by preventing T-cell activation and the synthesis of IL-2. This non-canonical effect of an AMPA receptor antagonist has implications for use in neuroinflammatory diseases.

Both treatments provide new approaches to treatment of alphavirus induced encephalomyelitis.

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# ABBREVIATIONS

**Ab** Antibody.

**ACI** (a-S, 5S)-a-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

**Ag** Antigen.

**AMPA** $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole.

**B6** C57BL/6 mice.

**BBB** Blood Brain Barrier.

**BHK** Baby Hamster Kideny.

**BPTES** Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide.

**BrdU** Bromodeoxyuridine.

**BSA** Bovine Serum Albumin.

**CFSE** Carboxyfluorescein Succinimidyl Ester.

**CHIKV** Chikungunya virus.

**CNS** Central Nervous System.

**CSF** Cerebrospinal Fluid.

**DAG** Diacylglycerol.

**DMEM** Dulbecco's Modified Eagle's Medium.

**DON** 6-Diazo-5-oxo-L-norleucine.

**EAE** Experimental Autoimmune Encephalomyelitis.

**ELISA** Enzyme-Linked Immunosorbent Assay.

**FBS** Fetal Bovine Serum.

**GLS1** Glutaminase isoform 1 (Kidney-type glutaminase).

**HBSS** Hank's Balanced Salt Solution.

**i.c.** intracerebral.

**i.n.** intranasal.

**i.p.** intraperitoneal.

**IFN-$\gamma$** Interferon-Gamma.

**Ig** Immunogolbulin.

**IHC** Immunohistochemistry.

**MS** Multiple sclerosis.

**NIH** National Institutes of Health.

**NMDA** N-Methyl-D-aspartate.

**NSV** Neuroadapted Sindbis Virus.

**PBS** Phosphate Buffered Saline.

**PFU** Plaque Forming Units.

**PMA** Phorbol 12-myristate 13-acetate.

**RPMI** Roswell Park Memorial Institute medium.

**SCID** Severe Combined Immune Deficiency.

**SINV** Sindbis Virus Virus.

**TNF-$\alpha$** Tumor necrosis factor alpha.

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# INTRODUCTION

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## 1.1 Introduction

Viral encephalomyelitis is inflammation of the brain and spinal cord as a result of viral infection. A range of RNA and DNA viruses can cause viral encephalitis (Table 1.1) [67]. Damage to CNS tissues can be mediated directly by the virus, indirectly by the immune system in response to viral infection, or a combination of both. Encephalitic viruses transmitted by arthropod vectors are an emerging public health threat [63]. Arboviral encephalitis is relatively uncommon and geographically restricted, but can lead to death and leave survivors with permanent neurological deficits in survivors. Presently, there are no treatments for arboviral encephalitis other than basic supportive care.

### 1.1.1 Arboviruses

Arthropod-borne viruses (arboviruses) are causes of acute encephalitis vectored by mosquitoes, ticks, and sandflies [66]. Arboviruses that cause encephalitis in humans are primarily RNA viruses from the Togaviridae (genus: Alphavirus) , Flaviviridae (genus: Flavivirus), Bunyaviridae (genus: Orthobunyavirus), and Reoviridae (genus: Coltivirus) families. Encephalitic arboviruses are generally maintained in a natural cycle between reservoir hosts (birds, small rodents) and arthropod vectors and can be transmitted to incidental hosts (do not amplify virus to promote transmission) such as humans and domestic animals where they can cause clinical illness [63]. However, some arboviruses establish an urban cycle in which humans develop sufficient viremia to transmit the virus to competent urban arthropod vectors. Additionally, domestic animals can also occasionally serve as amplifying hosts resulting in increased risk of transmission to humans.

Due to its vectored transmission by arthropods, arboviral encephalitis tends to

be seasonal and geographically restricted to endemic areas [66]. However, climate change and human encroachment resulting in the redistribution and introduction of competent arthropod vectors, movement of human populations and domesticated animals, and viral evolution play key roles in the geographic expansion and frequency of arboviral diseases [63, 66, 139, 187].

This dissertation will focus on alphavirus-induced encephalomyelitis and will use the prototypic alphavirus, Sindbis virus (SINV), in a mouse model system to study strategies to treat acute alphavirus encephalomyelitis.

### 1.1.2 Alphavirus Encephalomyeltitis

Viruses in the Alphavirus genera are geographically categorized into New World (Americas) and Old World (Europe, Asia, Africa, Australia) alphaviruses. The New World alphaviruses, such as eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), and eestern equine encephalitis (WEE) viruses, tend to be neurovirulent and are a reemerging/emerging source of mortality and morbidity in humans and domestic animals, specifically horses, in the Americas. The Old World alphaviruses such as Sindbis (SINV), Ross River (RRV), Semliki Forest (SFV) and Chikungunya (CHIKV) viruses do not cause life-threatening disease in humans but can cause debilitating polyarthritis resulting in significant morbidity. However, recent case reports suggest that Old World alphaviruses, in particular CHIKV, can also be associated with encephalitic disease in humans [30, 58, 127, 163].

Humans and domestic animals are dead-end (incidental) hosts for most alphaviruses, because they do not develop sufficient viremia to infect the arthropod vectors and sustain the transmission cycle [63]. However, these incidental hosts can develop significant clinical diseases when infected. In particular, infection with EEE results in a

| | Virus Family | Genus | Species |
|---|---|---|---|
| **Arboviruses** | *Togaviridae* | *Alphavirus* | Eastern equine† |
| | | | Western equine† |
| | | | Venezuelan equine† |
| | | | Sindbis* |
| | | | Chikungunya* |
| | | | Semliki Forest* |
| | | | O'Nyong-Nyong* |
| | | | Ross River* |
| | *Flaviviridae* | *Flavivirus* | St. Louis |
| | | | Murray Valley |
| | | | West Nile |
| | | | Japanese |
| | | | Dengue |
| | | | Tick-borne complex |
| | *Bunyaviridae* | | LaCrosse |
| | | | Rift Valley |
| | | | Toscana |
| | *Reoviridae* | | Colorado tick fever |
| | *Paramyxoviridae* | *Paramyxovirus* | Mumps |
| | | *Morbillivirus* | Measles |
| | | *Henipavirus* | Hendra |
| | | | Nipah |
| | *Arenaviridae* | *Arenavirus* | Lymphocytic choriomeningitis |
| | | | Machupo |
| | | | Junin |
| | *Picornaviridae* | *Enterovirus* | Poliovirus |
| | | | Coxsackievirus |
| | | | Echovirus |
| | *Retroviridae* | *Lentivirus* | HIV |
| | | *Deltaretrovirus* | HTLV-I |
| | *Rhabdoviridae* | *Lyssavirus* | Australian bat lyssavirus |
| | | | Rabies |
| | *Polyomaviridae*§ | *Orthopolyomavirus* | JC Virus |
| | *Herpesviridae*§ | *Herpesvirus* | Herpes simplex virus types 1 and 2 |
| | | | Epstein-Barr virus |
| | | | Cytomegalovirus |

**Table 1.1:** *List of viruses that can cause encephalomyelitis in humans.* This is not an exhuastive list.
*Old World alphavirus - not encephalitic in humans
†New World alphavirus - encephalitic in humans
§DNA virus family Adapted from: [157]

| | Alphavirus | Geographic Distribution | Clinical Outcome |
|---|---|---|---|
| **New World** | Eastern Equine Encephalitis (EEE) | Americas | fever,encephalitis |
| | Western equine Encephalitis (WEE) | North America | fever,encephalitis |
| | Venezuelan Equine Encephalitis (VEE) | Americas | fever, encephalitis |
| **Old World** | Sindbis (SINV) | Africa, Europe, Australia | fever,rash, arthritis |
| | Ross River (RRV) | Australia, South Pacific | encephalitis |
| | Semliki Forest(SFV) | Africa | |
| | Mayaro(MAY) | Africa | fever, rash, arthritis |
| | O'nyong-nyong(ONNV) | South America | fever, rash, arthritis |
| | Chikungunya(CHIKV) | Africa, India, South East Asia | fever, rash, severe arthritis |

**Table 1.2:** *Old and New world Alphavirus examples*

high proportion of fatal encephalitis in humans (50-70% case fatality) and horses (estimated 70-90% case fatality) [194]. There are no current treatments for alphavirus induced encephalitis other than supportive care. Veterinary vaccines do exist and human vaccines are in early clinical development. There is a current lack of targeted pharmacological countermeasures and there has been difficulty in making cross-strain vaccines that generate robust longterm immune responses [22].

### 1.1.3 Bioterrorism

Alphaviruses are very easily grown to high titers in cell culture in a variety of common transformed and primary cell lines. Additionally, alphaviruses, such as VEE and EEE, are stable and highly infectious even when aerosolized [50, 136, 143, 148, 158]. Moreover, the alphaviral genome lends itself to easy genetic manipulation by popular recombinant DNA methods. The encephalitic New World alphaviruses, EEE, VEE, and WEE are particularly dangerous threats due to their ability to cause significant

morbidity and mortality in humans. These characteristics have motivated weaponization of encephalitic alphaviruses. Due to their weaponization potential, VEE, WEE, and EEE are considered Category B agents by the Centers for Disease Control (CDC). In the past, both the United States and the former Soviet Union armed forces have had serious offensive biological weapons programs that included the weaponization of encephalitic alphaviruses [121]. The Biological Weapons Convention of 1972 dismantled the United States's offensive biological warfare program. However, research into defensive countermeasures against encephalitic alphaviruses continue.

### 1.1.4 Alphavirus Replication

Alphavirus is a genus in the family Togaviridae, that consists of viruses with enveloped, non-segmented, single stranded, positive sense RNA genomes of 11-12kb in size and icosahedral capsids. The message sense RNA genome (49S RNA) is single stranded with a 5' cap and a poly (A) tail with two open reading frames. The virus enters cells though receptor-mediated endocytosis via clathrin-coated pits. The 49S genomic RNA is released into the cytoplasm where translation of the first open reading frame encoding viral nonstructural proteins (nsP1, nsP2, nsP3, nsP4) as a polyprotein and proteolytic processing occur. The non-structural proteins assemble from the viral replication complex and generate the full length minus-sense RNA which serves as template for the generation of the message sense subgenomic (26S) RNA encoding the structural polyprotein and genomic (49S) RNA. The subgenomic (26S) RNA is translated as a polyprotein (Capsid-pE2-6K-E1). The capsid is released cotranslationally and assembles the icosahedral nucleocapsid core by associating with the nascent 49S genomic viral RNA via its N-terminal amino acid residues. The pE2-6K-E1 polyprotein is translocated in the endoplasmic reticulum where it undergoes

further proteolysis and posttranslational modifications to generate the mature viral envelope proteins. The E1 and pE2 membrane glycoproteins form heterodimers and are transported to the plasma membrane where pE2 is processed to E2 and E3 and the cytoplasmic domain of E2 interacts with the nucleocapsid core to initiate budding. The E1-E2 heterodimers trimerize to form spikes on the virus surface. Progeny virus is released from the plasma membrane with E1 and E2 embedded in the host lipid membrane bilayer. During infection the E2 protein is responsible for attachment while E1 is responsible for fusion with the cell membrane. No definite cellular receptors or family of receptors have been identified for alphaviruses. Because alphaviruses have a wide host range (birds, rodents, primates, and mosquitoes) it has been hypothesized that they might utilize either an unknown evolutionarily conserved receptor or a wide range of cellular receptors [93].

### 1.1.5 Sindbis Virus

Sindbis virus (SINV), the prototypic alphavirus was first isolated from mosquitoes in Sindbis, Egpyt in 1952 (AR339 strain) [164]. Wild type SINV causes fever, rash, and arthritis in humans. It has a wide geographic range, but disease is only recognized in South Africa and northern Europe. In mice, SINV causes acute encephalomyelitis. In vivo, a range of factors including age, genetic background, virus strain, route of inoculation, and immune competency determine the outcome and course of SINV infection in mice. SINV's trophism for neurons after intracranial or intranasal inoculation makes it an ideal model for study of neurovirulence and the immune response to CNS alphaviral infection. Additionally, this neurotrophism provides an interesting model to study how neurons facilitate and respond to viral infection in general. Mice usually clear infectious virus within a week after innoculation while viral RNA

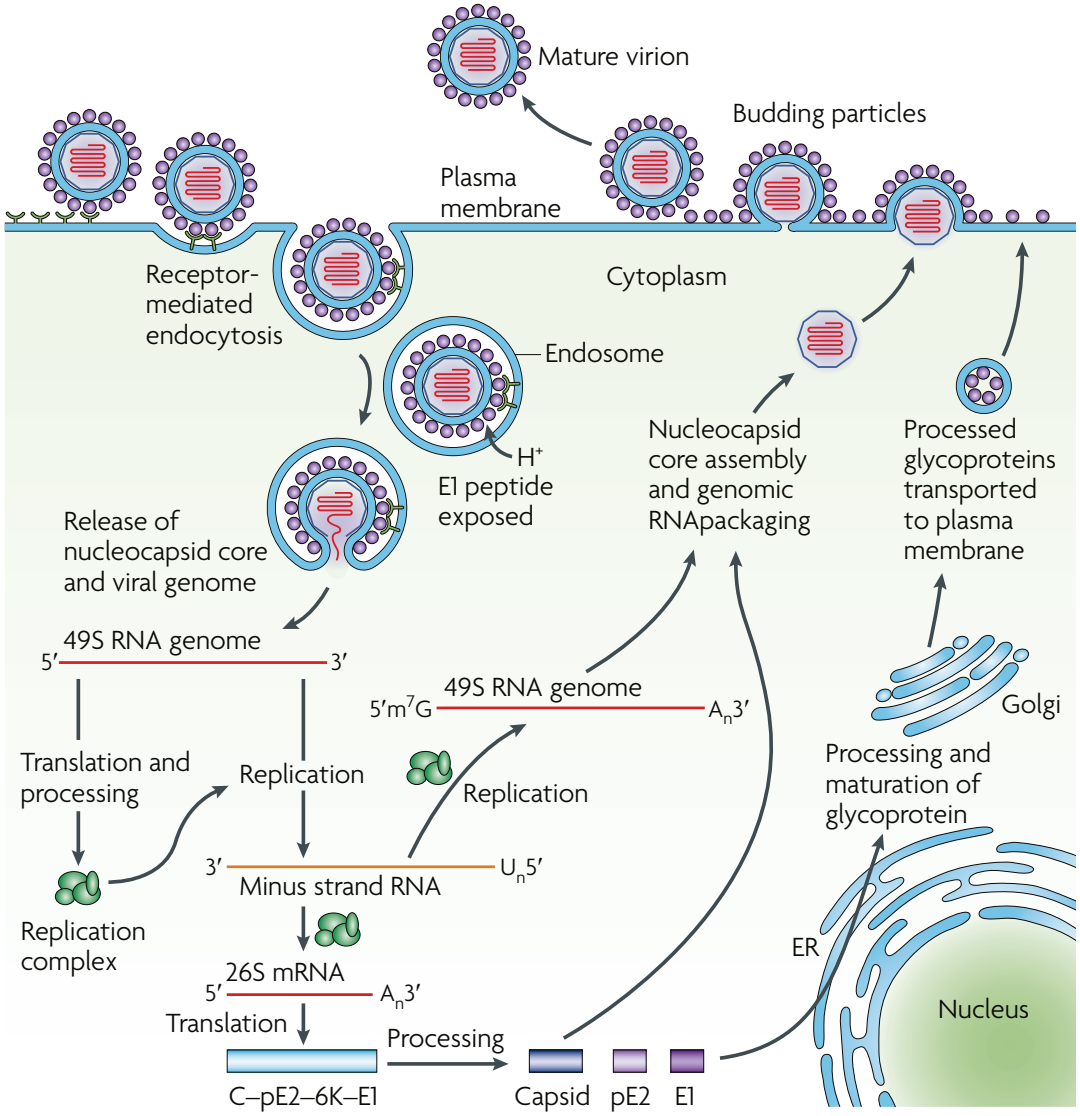


**Figure 1.1:** *Alphavirus replication.*

Source: [151]

persists for months.

SINV has a wide host range and can be cultured in a wide variety of transformed and primary cell lines. SINV infection of vertebrate cell lines leads to the shutdown of host protein synthesis, loss of membrane potential, and apoptosis. The plus-strand RNA genome makes it amenable to genetic manipulation of cDNA clones of the virus genome. Virulent (NSV) and avirulent (TE) derivatives of the original AR3339 strain are used to study acute and long term consequences of alphaviral encephalitis. Avirulent derivatives allow us to follow mice over the course of months and understand host and viral factors that mediate clearance of infectious virus, understand viral RNA persistence in neurons, and recrudescence of viral replication, and long term sequelae. The virulent strain allows modeling of acute fatal viral encephalitis to determine mechanisms of neuronal death and approaches to treatment.

### 1.1.6 NSV - A Model for Acute Fatal Viral Encephalomyelitis

Neuroadapted Sindbis Virus (NSV, Figure 1.2), was derived by serial alternating intracranial passages of the wild type AR339 strain of SINV between suckling and weanling BALB/c mice [68]. Compared to AR339, NSV replicates to 10-50 fold higher titers in the brain and is virulent in both neonatal and weanling mice. Weanling mice develop fatal encephalitis after intracranial or intranasal infection. The increase in efficiency of replication in mature neurons and neurovirulence have been attributed to mutations in the E1 and E2 envelope glycoproteins [107, 175, 176]. BALB/c mice show an age-dependent resistance to NSV-induced fatal encephalitis while all ages of C57BL/6 mice are susceptible to fatal NSV-induced disease resulting in a consistent 100% mortality even when infected with a low dose of PFU [166].

In C57BL/6 mice, NSV replicates in neurons and reaches peak titers in the brain

and spinal cord earlier than in BALB/c mice. Adult C57BL/6 mice inoculated with NSV succumb to progressive paralysis within ten days after infection (Figure 1.2B) while adult BALB/c mice develop mild paralysis and recover from infection. The underlying genetic determinants for the differential susceptibility of BALB/c and C57BL/6 mice have been mapped to a region on Chromosome 2 in linkage studies but the responsible gene has not been identified [167].

Previous studies have shown that the immune response to NSV plays a major role in neuronal damage in adult B6 mice [90]. Survival is improved in mice deficient in CD4+ or CD8+ T-cells and after treatment with drugs that inhibit the inflammatory response to infection [65, 89, 125, 147]. Animal death has been closely associated with the death of brain and spinal cord neurons [21, 74, 90, 99, 102, 141, 179].

### 1.1.7 Noncytolytic clearance of Sindbis virus from neurons

Adult neurons are post-mitotic, terminally differentiated cells with complex intracellular connections. Viral replication or the immune response to viral infection can cause neuronal damage. In the periphery, virus is often cleared through the cytolytic mechanisms of CD8+ cells. Because lysis of neurons can be detrimental to the host, non-cytolytic mechanisms are necessary to control viral replication and clear infectious virus without neuronal damage if the host is to recover [67]. Studies using an avirulent strain of SINV have highlighted factors that mediate non-cytolytic clearance of SINV in neurons. Both antibody against the E2 glycoprotein and IFN-$\gamma$ produced by CD8+ and CD4+ cells contribute to the non-cytolytic clearance of virus from neurons [6, 15, 16, 43, 101, 116]. In the absence of IFN-$\gamma$ and SINV-specific antibody adult neurons become persistently infected.

Bi-valent antibody is required for effective clearance of virus from neurons and

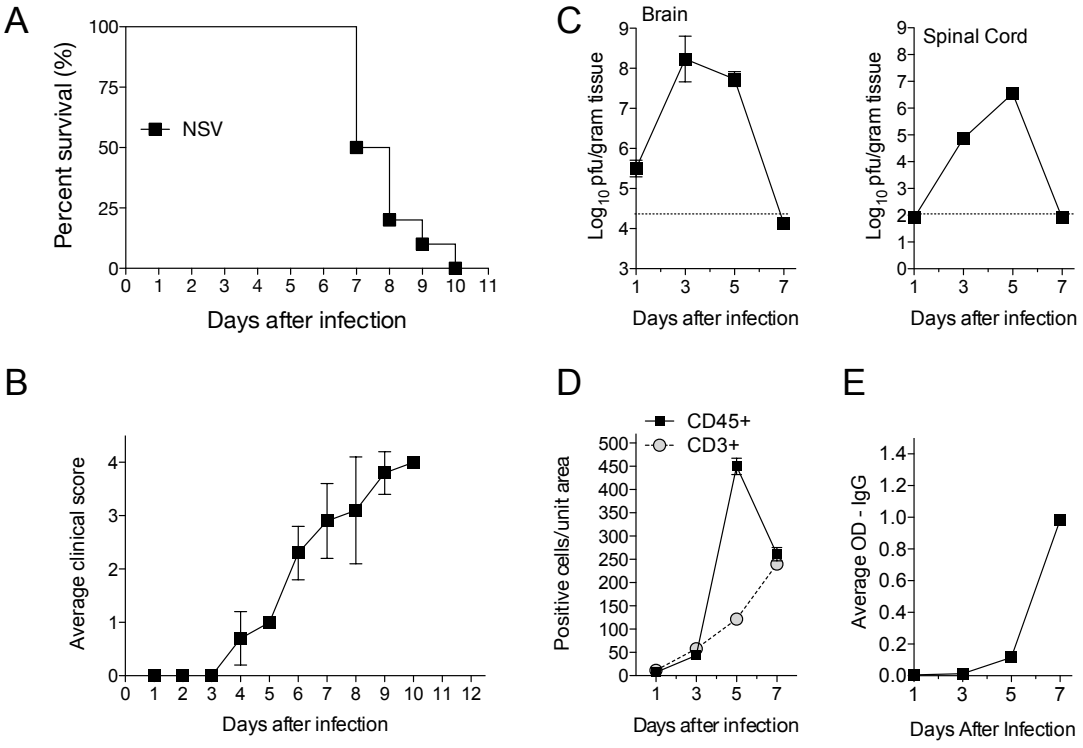


**Figure 1.2:** *Profile of NSV infection in adult C57BL/6 mice.*
Mice (4-6 weeks)infected i.c. with 1000pfu of virus. **(A)** NSV survival curve **(B)** Clinical score criteria: (0) No clinical signs, (1) mild weakness (2) one hind limb paralyzed, (3), both hind limbs paralyzed, (4) death. **(C)** NSV viral titers in the brain and spinal cord (dotted line represents limit of detection). **(D)** Profile of the leukocyte infiltration in the CNS measured by immunohistochemitry **(E)** Serurm IgG. *Figures adapted from: [65]

antibody-mediated clearance is independent of complement and leukocytes [69, 178]. In vitro studies have shown that treatment of SINV-infected cells with monoclonal antibody against the E2 surface glycoprotein inhibits intracellular viral replication, restores host protein synthesis, membrane potential, and the ability to respond to type I interferons [43, 44, 67]. Furthermore, local production of antibody is necessary for long term control of SINV replication in neurons. In immune competent mice infectious virus is cleared from the brain and spinal cord by day 7 post infection. However, viral RNA persists and is slowly cleared over the course of months and is facilitated by CD8+ T-cells [89, 100, 177]. Passive transfer of SINV-specific antibodies or hyperimmune serum into persistently infected, immunodeficient adult mice results in a temporary clearance of infectious virus followed by recrudescence of infectious virus once antibodies levels wane [90, 101].

Neurons express IFN-$\gamma$ receptor subunits and respond to IFN-$\gamma$ through the Jak/Stat-1 pathway. However, receptor expression and the ability to respond to IFN-$\gamma$ varies between different types of CNS neurons [16]. While SINV-specific antibody can clear infectious virus from both brain and spinal cord neurons, IFN-$\gamma$ alone can clear infectious virus from spinal cord neurons but not from hippocampal or cortical neurons [6, 17]. Treatment of SINV-infected differentiated neuronal cells with IFN-$\gamma$ increases cell survival, restores host protein synthesis, and limits viral replication by decreasing viral transcription and protein synthesis [16, 17]. Presently, the down stream effector(s) of antibody or IFN-$\gamma$ in neurons that limit SINV replication are not known, but the induction of nitric oxide (NO) may be important for NSV infection [174]. Antibody and IFN-$\gamma$ work together synergistically to control SINV replication throughout the brain and spinal cord and prevent reactivation of SINV [17].

### 1.1.8 The Immune Response to Sindbis Virus Infection

#### The Innate Immune Response

Innate cytokines play an important role in SINV spread and replication though the production of IFN-$\alpha/\beta$ and IFN-inducible genes that inhibit virus replication [19]. Peak IFN-$\beta$ levels are coincident with peak SINV titer in the brain around Day 3 post infection (Figure 1.2C). Mice deficient in interferon responses develop fatal disease and deficiency of IFN-$\beta$ results in 10-fold higher titers in the brain and spinal cord early in infection [17]. The production of type I interferons is IRF7 (Interferon Regulatory Factor 7)-dependent and mice defective in IRF7 succumb to NSV infection earlier and have 10-1000 fold higher viral titers in the brain and spinal cord than wild type mice [52, 53].

Studies suggest that activated microglia, the CNS's resident macrophages, might be the initial source of interferons (IFN-$\alpha$), inflammatory cytokines (IL12p40, IL1$\beta$, TNF-$\alpha$) and chemokines (CCL2, CCL5, CCL3, CXCL10). The mechanism by which NSV activates microglia is still under study. A report by Esen, et al. using in vitro primary microglial cultures showed that NSV can activate multiple pattern recognition receptors and that type I interferon production was independent of TLR signaling but dependent on IRF7 (MyD88 KO and TLR3 KO mice show no difference in mortality compared to wild type mice and can produce type I interferons) [52, 53]. Infiltrating myeloid cells can also produce type I interferons, inflammatory cytokines and chemokines. The pharmacological depletion of circulating monocytes by clodronate-loaded liposomes or the depletion of neutrophils does not alter the course of NSV-induced fatal disease [52, 95]. Therefore, the early production of type I interferons by CNS resident microglia might be sufficient to limit and control viral replication

with NSV fatal disease might be mediated by mechanisms independent of infiltrating myeloid cells.

Infection with NSV also results in the production of innate inflammatory cytokines IL-1$\beta$, TNF-$\alpha$, and IL-6 in the CNS [188]. These cytokines are produced early by microgila and subsequently by infiltrating leukocytes and act on neuronal, glial, and endothelial cells of the blood brain barrier. The blood brain barrier (BBB) is a highly selective permeability layer that separates the cellular and non-cellular soluble components of the blood from CNS parenchyma. It is composed of capillary endothelial cells joined by tight junctions and supported by astrocytes and pericytes. It supports the facilitated passage of essential nutrients, such as glucose, and passive diffusion of small hydrophobic molecules and gases. However, during inflammation, traumatic brain injury, or ischaemia the BBB becomes compromised allowing the entry of potentially neurotoxic substances into the CNS parenchyma.

During acute inflammation, inflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ contribute to increased permeability of the BBB and to neuronal and astrocyte dysfunction. In addition, these cytokines prime the BBB for leukocyte entry by upregulating adhesion molecules on endothelial cells that facilitate recruitment of leukocytes into the parenchyma [78]. Hence, its not surprising that mice defective in IL-1$\beta$ or treated with antibody against IL-1$\beta$ receptor are protected against fatal NSV infection, have less paralysis, and neuronal cell death [79, 103, 141]. Adult BALB/c mice which are resistant to NSV fatal disease show lower levels of IL-1$\beta$ in the spinal cord during NSV infection than susceptible C57BL/6 mice [141]. Similarly, mice deficient in TNF-$\alpha$ have improved survival after NSV infection and less death of brain and spinal cord neurons [21]. The contributions of IL-1$\beta$ and TNF-$\alpha$ to NSV-induced paralysis has

been attributed to their ability to perturb the clearance of the CNS's primary excitatory neurotransmitter, glutamate (see Glutamate Excitotoxicity section) [21, 141]. In vitro studies have also implicated both IL-1$\beta$ and TNF-$\alpha$ in the up regulation of glutaminase, the enzyme involved the the production of glutamate [191]. Hence, pharmacological inhibition of early microglial cell activation improved survival after NSV infection, protected spinal cord neurons, and limited severe paralysis. This result was attributed to lower levels of IL-1$\beta$ and TNF-$\alpha$ and improved clearance of glutamate as a consequence [21, 79, 140, 141]. Furthermore, delaying treatment decreased the efficacy of these interventions in protection against NSV induced mortality [79, 140]. These studies show that the early innate response plays an important role in the outcome of NSV infection.

**The Adaptive Immune Response**

While type I interferons control viral replication in the CNS, the adaptive immune response is required for clearance of infectious virus. The adaptive immune response is induced in the superficial and deep cervical lymph nodes and peak proliferation of lymphocytes is observed around day 5 post infection. SINV antigen presented to antigen-specific lymphocytes in the lymph nodes results in the clonal expansion of IFN-$\gamma$ producing anti-viral CD4+/CD8+ T-cells and antibody-producing B-cells. Early during infection B-cells produce low affinity IgM antibodies which is replaced by production of IgG by day 7 (Figure 1.2E). Lymphocytes exit the lymph node and reach peak CNS infiltration by day 7 post infection (Figure 1.2D). The onset of paralysis around days 4-5 is coincident with the beginning of anti-viral T-cell infiltration and the beginning of clearance of infectious virus from the brain and spinal cord (Figure 1.2B, C, D). By day 7 mice clear infectious virus, develop severe hindlimb paralysis,

and start to die (Figure 1.2A-C).

The adaptive anti-viral T-cell immune response contributes to paralysis and fatal CNS disease in NSV infection [147]. Susceptible C57BL/6 mice infected with NSV have higher survival in TCR-$\alpha/\beta$-deficient mice suggesting a contribution of T-lymphocytes to NSV-induced pathology. Also, mice deficient in class I antigen presentation ($\beta$2 microglobulin, TAP1) or CD4+ T cells have improved survival after NSV infection. NSV-infected SCID mice develop persistent infection [188]. In addition to being a source of IFN-$\gamma$, CD8+ T-cells play an important role in clearing viral RNA from neurons. Mice deficient in CD8+ cells have a lower rate of viral RNA clearance compared to immunocompetent mice [89]. Recent studies have shown a role for pathogenic Th17 cells in fatal NSV infection [95]. In total, studies suggest that fatal disease is mediated by infiltrating anti-viral T-lymphocytes.

### 1.1.9 SINV-induced death in mature neurons.

SINV can infect and induce apoptosis in a range of cell types. Apoptosis is initiated during virus entry and doesn't require viral replication [84]. Mature neurons are less susceptible to Sindbis virus-induced apoptosis than immature neurons. In the absence of an immune response, mature neurons become persistently infected. Though mature adult neurons are more resistant to Sindbis virus-mediated apoptosis, these post-mitotic and irreplaceable cells succumb to damage during clearance of NSV infection suggesting that the mechanisms mediating clearance of a virulent virus are also responsible for neurological damage with evidence of both apoptosis and necrotic neuronal death in infected and uninfected neurons. Glutamate excitotoxicity is a potential contributor to the bystander death of uninfected neurons during NSV infection. [39,65,124,125]. Interestingly, neurons in the brain show morphological evidence

of both necrotic and apoptotic death during NSV infection, while spinal cord motor neurons exclusively show signatures of necrosis [74]. This is further confirmed by the inability to find any evidence of caspase-3 cleavage in spinal cord neurons and the inability of viruses overexpressing anti-apoptotic proteins to protect motor neurons from death during NSV infection [74, 88].

### 1.1.10 Glutamate excitotoxicity

The amino acid glutamate is the principal excitatory neurotransmitter in the mammalian CNS where it is synthesized and stored in the neuronal cytosol and synaptic vesicles in millimolar concentrations. In the CNS, glutamate is not only important in neuronal synaptic transmission but also in nervous system development, and metabolism [126]. Extracellular concentrations of glutamate in the synaptic cleft are kept low (nanomolar ranges) by specialized glutamate transporters on neurons and astrocytes to prevent involuntary signaling events and to remove excess glutamate after the completion of a signaling event, returning glutamate in the synaptic cleft to homeostatic levels (Figure 1.3) [3]. An intact adult blood brain barrier restricts transport of glutamate and glutamine into the CNS. The carbon backbone for glutamate in the CNS is primarily derived from the catabolism of glucose molecules, neuron's primary energy source. Glutamate's glucose-derived carbon backbone is continuously recycled between glutamine and glutamate by the enzymes glutaminase (GLS1) and glutamine synthetase (Figure 1.3). In addition to being the precursor to glutamate, glutamine also supports the nutrient needs of neurons in hypoglycemic conditions and is used to carry excess ammonium out of the CNS [98].

The accumulation of excess glutamate in the extracellular space as a consequence of neuronal damage due to CNS trauma, neurodegenerative diseases, infections, and

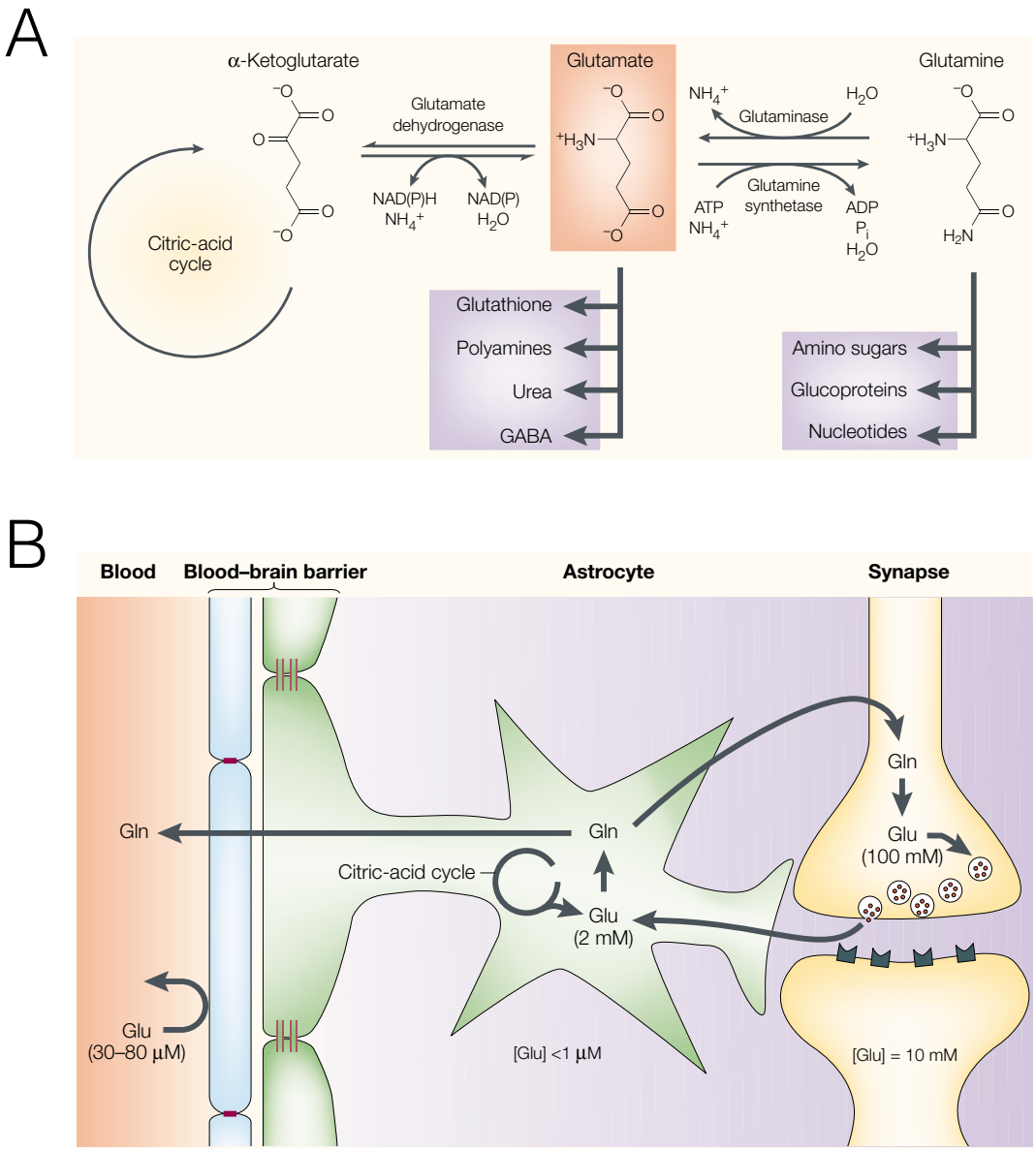


**Figure 1.3:** *Glutamate recycling in the CNS.*
**(A)** Key steps in glutamine (Gln) metabolism. **(B)** After a signaling event, excess glutamate (Glu) is taken up astrocytes and recycled is recycled back to glutamine by glutamine synthetase and released into the extracellular space for uptake by neurons. Neurons uptake the glutamine and regenerate the neurotransmitter glutamate by glutaminase (GLS1). Glutamine is also used to carry excess ammonium out of the CNS [98]. Figures from: [126]

deregulation of glutamate clearance results in glutamate-mediated excitotoxicity. Activation of ionotropic glutamate receptors (AMPA, NMDA, and kainate) by excess glutamate results in the promiscuous activation of downstream signaling cascades culminating in neuronal damage and further release of intracellular glutamate affecting nearby neurons. Compared to hippocampal and cortical neurons, spinal cord motor neurons are more susceptible to damage as a result of glutamate excitotoxicty, possibly due to the abundance of calcium permeable AMPA receptors on motor neurons [34, 125, 181]. A range of acute and chronic neurologic diseases, including viral encephalitis, have been implicated in this pathology.

Innate cytokines, IL-1$\beta$ and TNF-$\alpha$, inhibit glutamate clearance by downregulating the CNS's primary glutamate transporter, GLT-1, on astrocytes during NSV infection [21, 141]. BALB/c mice, resistant to NSV fatal infection, have 3-4 times higher levels of GLT-1 expression in spinal cord motor neurons (this was coincident with 4x lower IL-1$\beta$ levels) compared to susceptible C57BL/6 mice [39, 141]. Similar findings were reported for TNF-$\alpha$ during NSV infection. Glutamate clearance was significantly reduced in the spinal cords of WT mice compared to TNF-deficient mice during NSV infection. Like IL-1$\beta$, TNF-$\alpha$ had detrimental effects on glutamate clearance during NSV infection [21]. Moreover, both IL-1$\beta$ and TNF-$\alpha$ have been implicated in the upregulation of the enzyme glutaminase (GLS1) in neuronal and microglial cells resulting in the excess production of glutamate which can result in autocrine and paracrine excitotoxicity [26, 191]. Additionally, infiltrating immune cells can provide other sources of extracellular glutamate [114, 195]. A compromised BBB as a result of direct damage or neuroinflammation can also leak serum glutamate and other neurotoxic molecules into the CNS parenchyma affecting normal neuronal function.

AMPA and NMDA receptors are ionotropic glutamate gated ion channels that are most implicated in glutamate excitotoxicity and pathology associated with neurodegenerative diseases of the CNS including viral encephalitis [5, 7, 12, 25, 26, 73, 77, 81, 94, 105, 120, 132–134, 155, 197]. In vitro studies with cultured primary cortical and spinal cord neurons infected with NSV have shown that bystander death of uninfected neurons via glutamate excitotoxicity can be ameliorated using NMDA and AMPA receptor antagonists [39, 124]. In vivo studies with NSV have shown that treatment with NMDA and AMPA receptor antagonists protects hippocampal neurons against neurodegeneration but only AMPA receptor antagonists protect both hippocampal and spinal cord motor neurons and rescue mice from NSV-induced fatal paralysis [125]. The effectiveness of the AMPA receptor antagonists, Talampanel and GYKI-52466 (GYKI), is due to a non-competitive inhibition of AMPA receptor signaling in neurons and to a non-canonical effect on inhibiting the induction of the peripheral immune response [65, 125]. Talampanel and GYKI-treated mice were protected against NSV-induced fatal paralysis, showed delayed viral clearance, and had a decreased peripheral immune response as measured by draining lymph node T-cell counts [65, 125]. These drugs worked to prevent glutamate excitoxicity by binding to AMPA receptors responding to excitotoxic glutamate on allosteric sites [4] and also inhibited peripheral T-cell responses by an unrecognized mechanism.

### 1.1.11 Significance

Presently there is no treatment for acute fatal alphaviral encephalomyelitis and the increase in disease due to mosquito-borne encephalitic viruses as a result of climate change and human migration pose an emergent public health risk. Moreover, survivors

of acute viral encephalitis are often left with neurological sequelae. Additionally, alphaviral encephalomyelitis also poses a possible bioterrorism threat. Treatments that are both neuroprotective and immunosuppressive could help reduce high case fatality rates and prevent permanent neurological damage in survivors of alphavirus encephalitis. This dissertation reports investigation of two treatments that hold promise for achieving these goals.

# CHAPTER 2

# EFFECTS OF GLUTAMINE ANTAGONIST DON, ON ACUTE FATAL NSV INFECTION.

**Contents**

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## 2.1 Introduction

Sindbis virus (SINV) is a mosquito-borne, enveloped, positive-strand RNA virus in the family Togaviridae and genus alphavirus. SINV causes rash and arthritis in humans and infects neurons to cause acute encephalomyelitis in mice [82] A range of factors including age, genetic background, virus strain, route of inoculation, and immune competency determine the outcome of infection in mice [89, 147, 166, 173]. A neuroadapted strain of SINV (NSV), obtained by serial passage in mice, causes progressive paralysis and death of adult C57BL/6 mice and provides a model for study of acute fatal viral encephalomyelitis [68]. Survival after NSV infection is improved in mice deficient in CD4+ or CD8+ T-cell responses and after treatment with drugs that inhibit the inflammatory response to infection [65,89,90,125]. The precise mechanism by which infiltrating immune cells responding to NSV infection cause damage in the CNS is not known, but both direct T cell-mediated cytotoxicity and glutamate excitotoxicity have been implicated. Because the adaptive antiviral immune response as well as glutamate excitotoxicity are potential mediators of neuronal death in NSV infection [65, 89, 90, 95, 125, 147], we investigated the protective effects of glutamine antagonists.

To determine if pharmacological inhibition of glutamine metabolism could protect mice from NSV-induced fatal paralysis, we treated mice with the glutamine antagonist, DON (6-diazo-5-oxo-norleucine). DON is a Streptomyces-derived antimetabolite that competitively and irreversibly binds to the active sites of many glutamine-utilizing enzymes, permanently inhibiting their catalytic activities [168]. DON was discovered in the 1950s and initially developed as a cancer chemotherapeutic [32, 91]. Clinical trials from the 1980s showed that DON treatment was effective

against tumor cells, but prolonged treatment resulted in substantial toxicity, so further development was not pursued [48, 49, 108, 159].

In our studies, acute treatment with relatively low doses of DON protected mice from acute fatal encephalomyelitis and inhibited the adaptive immune response to NSV infection. DON-induced suppression of the antiviral immune response resulted in decreased CNS inflammation and prevented infectious virus clearance. However, with the cessation of treatment, the appearance of the antiviral immune response coincided along with viral clearance and fatal encephalomyelitis, confirming the role of the immune response in disease.

## 2.2 Materials and Methods

### 2.2.1 Cell Culture and Drugs

BHK cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (pen), streptomycin (strep), and 2mM glutamine (Invitrogen). Primary lymphocytes were grown in DMEM supplemented with 10% dialyzed FBS, pen/strep, glutamine, and 50$\mu$M $\beta$-mercaptoethanol (Sigma). Glutamine-deficient DMEM was used in certain experiments. All cells were grown at 37℃ with 5% CO2. Glutamine antagonists, 6-diazo-5-oxo-L-norleucine (DON; Sigma) and Acivicin (ACI; Sigma) were solubilized in sterile PBS to prepare 100mM stock solutions. Working dilutions were made in media for in vitro experiments or in sterile PBS for in vivo experiments. Stock solutions were stored at -80℃ and fresh working solutions were made for each use.

### 2.2.2 Virus and plaque assay

NSV [68] was grown in BHK cells in DMEM supplemented with 1% FBS, pen/strep and glutamine. Supernatant fluid was collected 24h after infection, filtered through a 40$\mu$M filter, and stored in aliquots at -80℃. For plaque assays supernatant fluids and tissue homogenates (20%) were serially diluted in DMEM with 1% FBS, inoculated onto BHK cells, incubated at 37℃ for 1h, washed and overlaid with agar (1.2% Bactoagar, MEM, 1% FBS). After incubation for 48h at 37℃, cells were stained with neutral red and plaques were counted.

### 2.2.3 Animals, infection, treatment, and tissue harvest

Six-to-eight week old female C57BL/6J mice (Jackson Laboratory) were inoculated intracerebrally with 1000 pfu NSV in 20$\mu$L of HBSS or PBS under light isoflurane anesthesia. Mice were treated daily with 100$\mu$L of PBS or 0.3 or 0.6 mg/kg DON in 100-200$\mu$L PBS intraperitoneally from day 0 through day 7 after infection. Mice were scored daily for disease using the following criteria: (0) No signs of weakness, (1) mild weakness, hunched posture, (2) one hind limb paralyzed, (3) both hind limbs paralyzed, and (4) death. For tissue collection, mice were deeply anesthetized and blood was collected by cardiac puncture into serum separator tubes (BD Microtainer). Mice were then perfused with ice cold PBS. Brain, spinal cord and cervical lymph node tissues were collected and either used fresh for cell analysis or snap frozen and stored at -80℃ for plaque assays and RNA extraction. All studies were done in accordance with protocols approved by the Johns Hopkins University Animal Care and Use Committee.

### 2.2.4 qRT-PCR Analysis

RNA was extracted from frozen brains using the RNeasy Lipid Tissue Kit (Qiagen). Extracted RNA was diluted to 1$\mu$g/$\mu$L and 2$\mu$g were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA (2.5$\mu$L) was analyzed for the following mRNAs by TaqMan quantitative RT-PCR: IL-1$\beta$ (mm0434228_m1), IL-6 (mm00466190_m1), TNF-$\alpha$ (mm99999068_m1), IL-12, CCL2 (mm00441242_m1), CCL5 (mm01302427_m1), CXCL10(mm99999072_m1), IL-10 (mm00439616_m1), TGF-$\beta$, IL-4 (mm00445259_m1), IFN-$\gamma$ (mm00801778_m1), T-bet (mm00450960_m1), GATA3 (mm00484683_m1), FoxP3 (mm00475162_m1), and RoRc-$\gamma$ (mm01261022_m1) (Applied Biosystems). Data were acquired on a 7500 real time PCR machine (Applied Biosystems) and analyzed using Excel software (Microsoft). Data from all samples were normalized to rodent GAPDH (Applied Biosystems) and fold induction was calculated relative to RNA from brains of uninfected mice.

### 2.2.5 Histopathology and Immunohistochemistry

Deeply anesthetized mice were perfused with 20mL ice cold PBS before being perfused with 40mL of ice-cold 4% paraformaldehyde (PFA). Brains were harvested and cut into 2mm coronal sections that were fixed with 4% PFA overnight at 4℃. Sections were rinsed with cold PBS, embedded in paraffin, sectioned (10$\mu$m), and stained with hematoxylin and eosin (H&E) by the Johns Hopkins Hospital Pathology Laboratory. Coded H&E sections were scored by Dr. Victoria Baxter using the following criteria: (0) no inflammation, (1) one or two occasional inflammatory foci, (2) moderate foci in <50% of 10x fields, (3) moderate-severe foci in >50% of 10x fields. A score of +1 was given for abundant cellularity. For immunohistochemistry, sections

were heated at 58°C for 10 min and then rehydrated through xylene, 100% ethanol, 95% ethanol, 70% ethanol and deionized water. Sections were boiled in citrate buffer (10mM citrate pH 6 with 0.05% Tween 20) for 10 min, cooled and washed in PBS. Sections were blocked with 5% normal goat serum in Neuropore (Trevigen) for 30 min and then stained with antibodies against NSV (1:100, polyclonal), CD3 (1:100, SP7 clone, Abcam) or NeuN (1:50; Millipore), overnight at 4°C in a humidified chamber. Slides were washed with PBST (PBS with 0.05% Tween 20) and then incubated with appropriate fluorescently conjugated (Alexa 594 or Alexa 488, Invitrogen) secondary antibodies diluted in Neuropore (1:200, Trevigen) for 1h at room temperature (RT). Slides were washed in PBST, immersed in 0.1% Sudan Black in 70% ethanol for 20 min, and washed. Sections were mounted using ProLong Gold with DAPI (Invitrogen) for 24h in the dark at RT and images obtained using a Nikon 90i microscope with Volocity software. Cells were counted in 10 random fields per tissue section under 20x magnification and averaged.

### 2.2.6 Isolation and analysis of mononuclear cells from brain and cervical lymph nodes

Mononuclear cells were isolated from brain as described previously [135]. Briefly, brains were homogenized in isotonic Percoll (9 parts Percoll; GE Healthcare, 1 part 10x HBSS; Mediatech), treated with DNase I (Sigma) for 30 min at RT and layered onto a 30/70% discontinuous Percoll gradient. After centrifugation at 500xg for 30 min, mononuclear cells at the interface were collected. Lymph nodes were placed on a 40$\mu$m mesh filter in a cold petri dish containing RPMI and disassociated using the blunt end of a syringe piston.

For analysis by flow cytometry, cells were stained with Aqua Live/Dead dye (Invitrogen), washed with FACS buffer (PBS with 1%BSA and 2mM EDTA), blocked

with Fc Block (BD Biosciences) for 30 min and stained with fluorescently conjugated antibodies against mouse CD3 (APC-Cy7, 17A2 clone, BD Biosciences), CD45 (FITC, 30-F11 clone, BD Biosciences), CD4 (PerCPCy 5.5, RM4-5 clone, BD Biosciences), CD8 (PE-Cy7, 53-6.7 Clone, BD Biosciences), and CD19 (APC, 1D3 clone, BD Biosciences). Washed cells were resuspended in 400$\mu$L FACS buffer and 50 $\mu$L of CountBrite beads (Invitrogen) were added. Cells were analysed on a FACS Canto II flow cytometer (BD Biosciences) for calculation of absolute numbers of live cells.

### 2.2.7 BrdU Incorporation Assay

BrdU (Sigma) stock (50 mg/mL) was prepared in PBS and stored in aliquots at -80 ℃. BrdU (2mg in 200$\mu$L) was administered intraperitoneally on days 4 and 5 after NSV infection. After 8h superficial cervical lymph nodes were removed and viability determined as above. Cells were fixed, permeabilized (BD Cytofix/Cytoperm) and then incubated in nuclear staining buffer (0.5% Triton-X 100, 1%BSA, 2mM EDTA) for 10 min, fixed using Cytofix/Cytoperm buffer and then incubated in DNase (Sigma) (30$\mu$g/$10^6$ cells) for 1h at 37℃. Cells were stained with FITC-conjugated antibody to BrdU (BD Biosciences) for 20 min at RT, washed and analyzed on a FACS Canto II flow cytometer.

### 2.2.8 In vitro T-cell analysis

CD3+ lymphocytes were purified from the spleens of adult C57BL/6J mice using a Pan T-cell Isolation Kit (Miltenyi Biotec). For activation, flat bottom 96-well plates were coated with anti-CD3 (5 $\mu$g/mL, 145-2C11 clone, eBiosciences) and anti-CD28 (2.5 $\mu$g/mL, 37.51 clone, eBiosciences) for 2h at 37℃ and washed. Prior to culture, lymphocytes were stained with CFSE (Invitrogen) in PBS with 0.1% BSA for 5 min at 37℃, washed, and then added at a density of $10^5$ cells/well. Cells were cultured

in glutamine-free or complete (DMEM, 10% dialyzed FBS, 2mM glutamine, NEAAs, 25mM HEPES) media in the presence or absence of DON or ACI 5-20$\mu$M. Cells were stained for CD3, CD4 (PerCPCy 5.5, RM4-5 clone, BD Biosciences), and CD8 as described above. Viability was assessed at 12h by flow cytometry (BD FACS Canto II) using a violet fluorescent exclusion dye (Invitrogen) according to the manufacturer's protocol. T-cell proliferation was assessed at 72h by CFSE dilution.

### 2.2.9 Enzyme immunoassays

For detection of NSV-specific IgG, 96-well plates were coated overnight at 4°C with lysates from BHK cells infected with NSV or with uninfected BHK lysates diluted in coating buffer (50mM $NaHCO_3$ pH 9.6). Plates were washed with PBST (PBS with 0.05% Tween-20), blocked with 10% FBS in PBST for 1h at RT or overnight at 4°C and washed. Mouse serum samples diluted 1:100 in blocking buffer were added and incubated for 1h at RT. Plates were washed and incubated with horseradish peroxidase-conjugated antibody against mouse IgG (1:2000, Southern Biotech). Color was developed with TMB (3,3,5,5-tetramethylbenzidine) substrate solution (Sigma). After adding stop solution (2M $H_2SO_4$), optical densities (ODs) at 450nm were determined. OD values from wells coated with uninfected BHK lysates were subtracted to obtain NSV-specific OD values. For measurement of IFN-$\gamma$ and IL-2, supernatant fluids collected from culture media 24h after activation of CD3+ splenocytes with anti-CD3 and CD28 were assayed for IFN-$\gamma$ and IL-2 (R&D Systems). Brain homogenates diluted 1:2 were assayed for IFN-$\gamma$.

### 2.2.10 Statistical analysis

Statistical analysis was performed using Prism 5 software (GraphPad). Two-way ANOVA with Bonferroni post test was used for analysis of differences between treated

and untreated mice at different time points. Student's t-test was used to compare BrdU incorporation between groups. One-way ANOVA with Dunnett's post test was used to calculate significance for BHK viral titers and in-vitro T-cell experiments. Log-rank (Mantel-Cox) test was used to compare Kaplan-Meier survival curves. $P<0.05$ was considered significant.

## 2.3 Results

### 2.3.1 During treatment with DON, mice were protected from NSV-induced acute fatal paralysis

To determine if DON could protect mice from NSV-induced fatal paralysis, infected mice were treated daily from day 0 through 7 with either PBS or DON (0.3 mg/kg or 0.6 mg/kg) (Figure 2.1). Most PBS-treated mice showed signs of paralysis by day 6 (Figure 2.1A) and all died by day 12 with a median day of death of 8 (Figure 2.1B). During the period of treatment, DON-treated mice had lower clinical scores compared to PBS-treated mice (Figure 2.1A). However, once DON treatment was stopped, paralysis developed and most mice died. Therefore, DON treatment delayed the onset of clinical disease with a median day of death of 14 for the low dose (0.3 mg/kg) and 12 for the higher dose (0.6 mg/kg). In addition to DON we used Acivicin (ACI), a nonspecific glutamine antagonist that is a structurally distinct from DON. ACI was also effective in preventing acute NSV induced fatal paralysis when mice were treated with 1mg/kg daily i.p. ( Figure 2.2).

### 2.3.2 DON treatment did not affect NSV replication, but delayed virus clearance from the brain and spinal cord.

To determine if glutamine deprivation or DON treatment affected NSV replication, growth in BHK cells was assessed in vitro (Figure 2.3A). Cells were serum and glutamine starved for 24h to deplete intracellular glutamine stores, then infected with NSV at MOIs (multiplicity of infection) of 1 and 10, and cultured in either complete media (DMEM without glutamine, 1% FBS, 2mM glutamine, pen/strep), glutamine-deficient media (DMEM without glutamine, 1% dialyzed FBS, pen/strep), or complete media containing glutamine antagonist DON or ACI (100$\mu$M). Virus production was assessed at 24h. Neither glutamine deprivation nor treatment with glutamine antagonists affected NSV replication in vitro (Figure 2.3A).

To examine the effects of DON treatment on virus replication and clearance in vivo, brains and spinal cords were harvested and assayed for plaque formation on BHK cells. DON treatment had no effect on peak viral replication with similar viral titers in the brains and spinal cords of DON-treated and PBS-treated mice before day 5 (Figure 2.3B). PBS-treated mice showed decreasing viral titers on day 5, but DON-treated mice did not start to clear virus from brain until day 9, 2 days after drug treatment was withdrawn. On days 7 and 9, DON-treated mice had 1000 to 10,000-fold higher virus titers in the brain and spinal cord than PBS-treated mice (Figure 2.3B). Clearance of virus was associated with the onset of neurologic disease in both DON-treated mice and PBS-treated mice(Figure 2.1).

### 2.3.3 DON-treated mice have less CNS lymphocyte infiltration.

To determine the effects of DON treatment on leukocyte infiltration into the brains of NSV-infected mice, we isolated and counted brain leukocytes. Fewer leukocytes

infiltrated the brains of DON-treated mice compared to PBS-treated mice (Figure 2.4). Total leukocyte counts (CD45+) and T lymphocyte (CD3+) counts, as well as CD4+ and CD8+ T-cell and CD19+ B-cell counts were higher in PBS-treated mice on days 5 and 7.

Analysis of H&E-stained coronal brain sections showed less inflammation in DON-treated than PBS-treated mice on day 7 (Figure 2.5A,B). However, mononuclear cells were present in the brains of surviving mice on day 13, six days after cessation of DON treatment. A similar trend was seen with infiltrating CD3+ lymphocytes when assessed by immunofluorescent staining (Figure 2.6A, B) with few cells in the brains of DON-treated mice on day 7, but many cells on day 13. Cellular infiltration was co-incident with clearance of virus as measured by detection of NSV antigen by immunofluorescent staining (Figure 2.7A, B) and infectious virus by plaque assay (Figure 2.3B).

Transcriptional analysis by quantitative RT-PCR for T-cell subtype-specific transcription factors T-bet (Th1), FoxP3 (Treg), RoRc-$\gamma$ (Th17), and GATA3 (Th2) showed that all subsets were affected by DON treatment with the most marked effect on expression of T-bet and FoxP3 (Figure 2.6C).

### 2.3.4 DON-treated mice have lower levels of cytokine and chemokine mRNAs in brain

To investigate the effects of DON on inflammatory mediators in the CNS, brains from DON-treated and PBS-treated NSV-infected mice were assayed for pro-inflammatory (IL-1$\beta$, IL-6, TNF-$\alpha$, IL-12), chemotactic (CCL2, CCL5, CXCL10), and anti-inflammatory (IL-10, TGF-$\beta$, IL-4) cytokine mRNAs. PBS-treated mice had peak pro-inflammatory mRNA expression at day 5 (Figure 2.8A). IL-1$\beta$, IL-6, and IL-12 mRNA levels decreased to day 1 levels by day 7 while TNF-$\alpha$ mRNA levels remained high longer

(Figure 2.8A). DON-treated mice did not have an increase in pro-inflammatory cytokine mRNA levels during treatment, but, with the exception of IL-6, levels increased on days 9-11 after treatment was stopped(Figure 2.8A). Similarly, the brains of DON-treated mice had lower levels of anti-inflammatory cytokine (Figure 2.8B) and inflammatory chemokine (Figure 2.8C) mRNAs during treatment that increased after stopping DON.

### 2.3.5 DON-treated mice have lower levels of IFN-$\gamma$ in brain and NSV-specific antibody in serum

CNS-infiltrating virus-specific IFN-$\gamma$-producing lymphocytes and antibody to the E2 glycoprotein contribute to the clearance of infectious virus from the brain and spinal cord through non-cytolytic mechanisms [6,15,16,101]. Because clearance is delayed in DON-treated mice, we measured the effect of treatment on IFN-$\gamma$ mRNA and protein levels in the brain and NSV-specific antibody in serum (Figure 2.9). In PBS-treated control mice, IFN-$\gamma$ mRNA levels peaked on day 5 after infection, and IFN-$\gamma$ protein levels peaked on day 7. In DON-treated mice, neither IFN-$\gamma$ mRNA nor protein increased during treatment. However, increases occurred on days 11 and 13 (Figure 2.9A) or 5 or 6 days following cessation of DON treatment. Similarly, NSV-specific IgG was present in serum of PBS-treated mice on days 7-9 after infection, but mice treated with DON produced little antibody until after treatment was stopped (Figure 2.9B). The appearance of IFN-$\gamma$ in brain and antibody in serum was coincident with initiation of clearance of infectious virus in both PBS-treated and DON-treated mice (Figure 2.3B).

### 2.3.6 DON-treated mice have lower lymphocyte proliferation in the draining cervical lymph nodes after infection

The induction of the adaptive immune response to NSV infection of the brain occurs primarily in the superficial and deep cervical lymph nodes. To determine the effects of DON on the peripheral lymphoid cell responses to infection, superficial cervical lymph nodes were isolated from PBS-treated and DON-treated NSV-infected mice. Cells were stained for CD45, CD3, CD4, CD8 and CD19 and counted using a flow cytometer (Figure 2.10). Cell counts were analyzed 5 days after infection when they peaked in PBS-treated mice. The percentages of viable cells were similar (Figure 2.10A), as were the proportions of CD3, CD4, CD8 and CD19-positive cells (Figure 2.10B,C). However, there was little evidence of expansion of lymphocyte numbers in DON-treated mice (Figure 2.10D) and this affected all populations, suggesting generalized suppression of proliferation (Figure 2.10D-F).

To directly assess proliferation of cells in the draining lymph nodes, mice were pulsed with the thymidine analogue BrdU 4 and 5 days after infection. Proliferating cells incorporate BrdU during the S-phase of the cell cycle and BrdU positivity identifies cells that were proliferating during the labeling period. Lymph nodes were harvested 8h after the second dose on Day 5 and cells assessed for BrdU incorporation via flow cytometry (Figure 2.11A). DON-treated mice had a lower percentage (Figure 2.11B) and number (Figure 2.11C) of T-cells and B-cells incorporating BrdU than PBS-treated mice.

### 2.3.7 ACI treatment protected mice from NSV-induced acute fatal paralysis

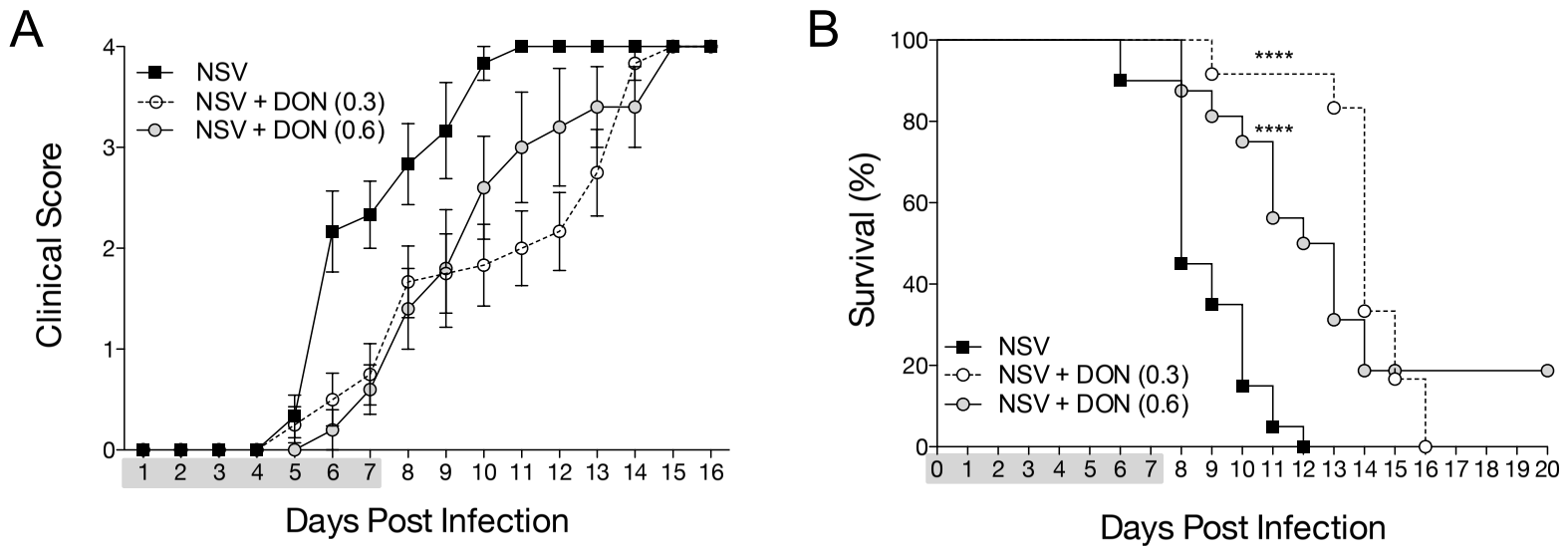


**Figure 2.1:** *DON treatment protects mice from NSV-induced acute disease.*
Six to eight week old C57BL/6 mice were infected with 1000 pfu of NSV intracerebrally and treated every 24 h intraperitoneally with glutamine antagonist DON (0.3 mg/kg or 0.6 mg/kg) or PBS vehicle (100 - 200$\mu$L) through day 7. **(A)** Clinical scores of treated and untreated mice (NSV, N=6; DON 0.3 mg/kg, N=6-12; DON 0.6 mg/kg, N=5). Clinical score criteria: (0) No clinical signs, (1) mild weakness (2) one hind limb paralyzed, (3), both hind limbs paralyzed, (4) death. **(B)** Mortality of treated and untreated mice (NSV, N=20; DON 0.3 mg/kg, N=12; DON 0.6 mg/kg, N=15). For untreated mice (NSV) median survival was 8 days while DON-treated mice had median survival time of 14 (0.3 mg/kg) and 12 (0.6 mg/kg) days. ****P <0.0001; Log-rank (Mantel-Cox) survival test.

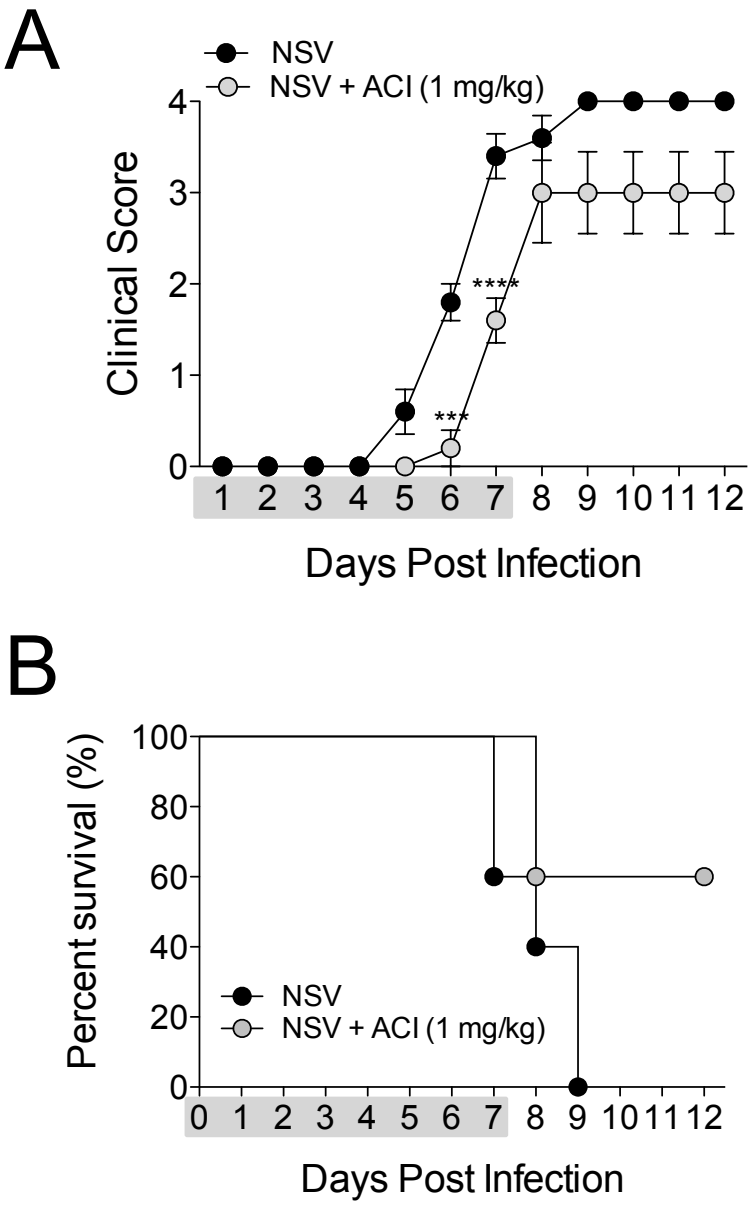


**Figure 2.2:** *Effect of glutamine antagonist, Acivicin (ACI), on outcome of NSV infection*

Acivicin (ACI) is a glutamine antagonist that is structurally distinct from DON but has similar mechanism of action. Like DON, ACI is non-specific glutamine antagonist. C57BL/6 mice (N=5 per group) were infected with 1000 pfu of NSV intracerebrally and treated every 24h intraperitoneally with ACI ( 1 mg/kg) or PBS vehicle (200$\mu$L) i.p. through day 7. ACI-treated mice had a lower clinical scores during treatment and 40% lower mortality after Day 9 compared to PBS treated mice. ***P<0.001 (Two-way ANOVA with Bonferroni post test) (A). Log-rank (Mantel-Cox) survival test (B).

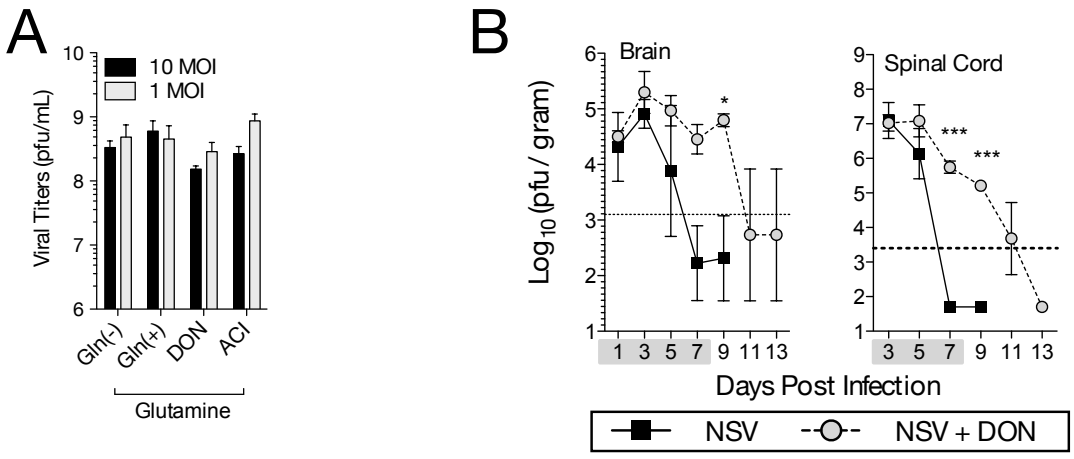


**Figure 2.3:** *Effect of glutamine deprivation on virus replication.* **(A)** Viral titers from NSV infection of BHK cells at MOIs of 1 and 10 in either complete media (Gln(+)), glutamine-deficient media (Gln(-)), or complete media with glutamine antagonists 6-Diazo-5-oxo-L-norleucine (DON 100$\mu$M) or Acivicin (ACI - 100$\mu$M). BHK cells were glutamine and serum-starved for 24 h before infection with NSV. Supernatant fluids were collected 24 h after infection and assayed by plaque formation on BHK cells. Difference between Glu(+) and other treatment groups were not significant either at MOI of 10 or 1. **(B)** Viral titers from the brains and spinal cords of PBS (NSV) and DON-treated (NSV + DON, 0.6 mg/kg-brain, 0.3mg/kg spinal cord) mice. Grey bars under the x-axis designate the drug treatment period. Error bars represent SEM of the geometric mean viral titers of three biological replicates **(A)** or three mice **(B)**. The dotted line represents the limit of detection of the plaque assay. Data are representative of at least two independent experiments **(B)**. *P<0.05; ***P<0.001 (One way ANOVA with Dunnetts post test **(A)**, Two-way ANOVA with Bonferroni post test **(B)**.

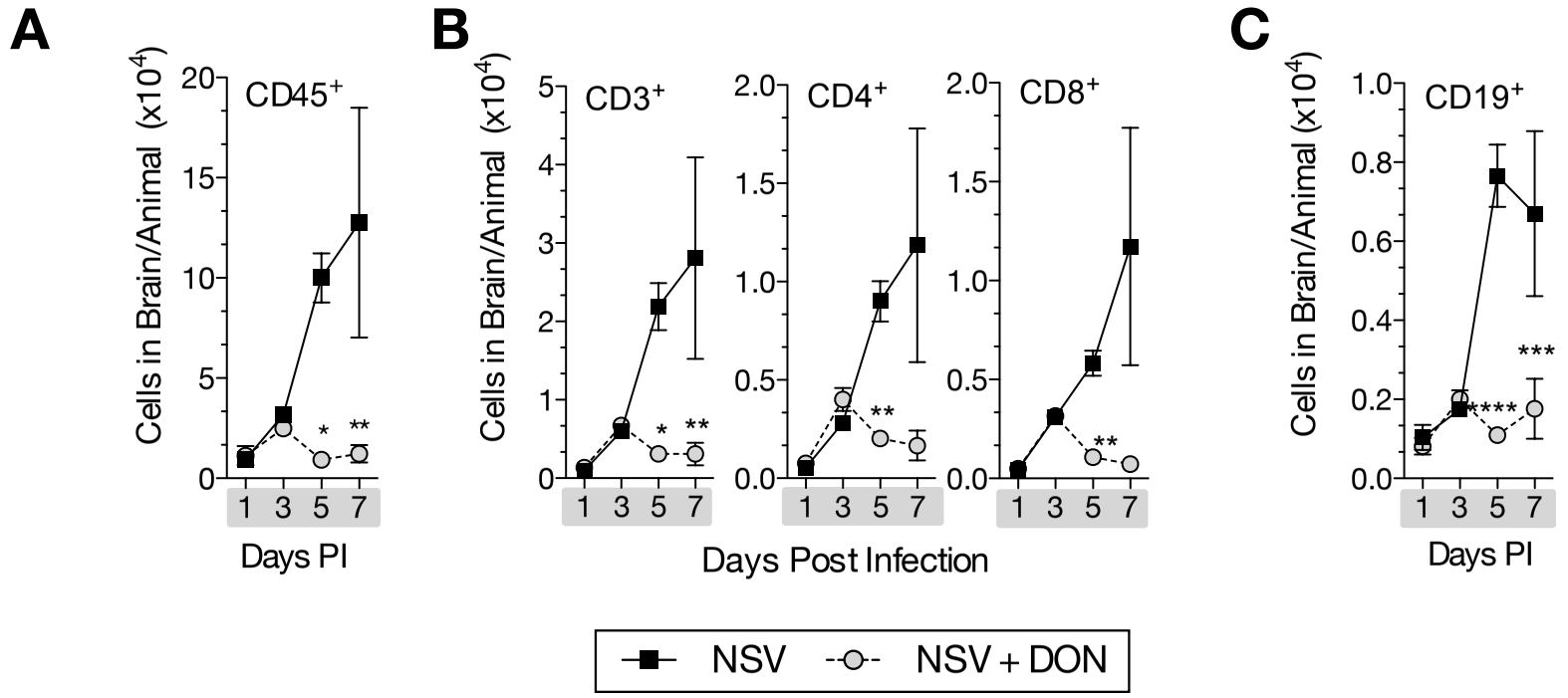


**Figure 2.4:** *DON-treated mice have decreased leukocyte infiltration into the brain.*
Inflammation in the brains of NSV-infected DON-treated and vehicle-treated mice (day 0 - 7) was evaluated by flow cytometry. Mononuclear cells were isolated from brains of individual mice by Percoll gradient centrifugation and numbers of **(A)** CD45+, **(B)** CD3+, CD4+, CD8+ and **(C)** CD19+ cells/brain determined. Data are representative of at least three independent experiments. *P<0:05; **P<0:01; ***P<0.001 (Two-way ANOVA with Bonferroni post test).

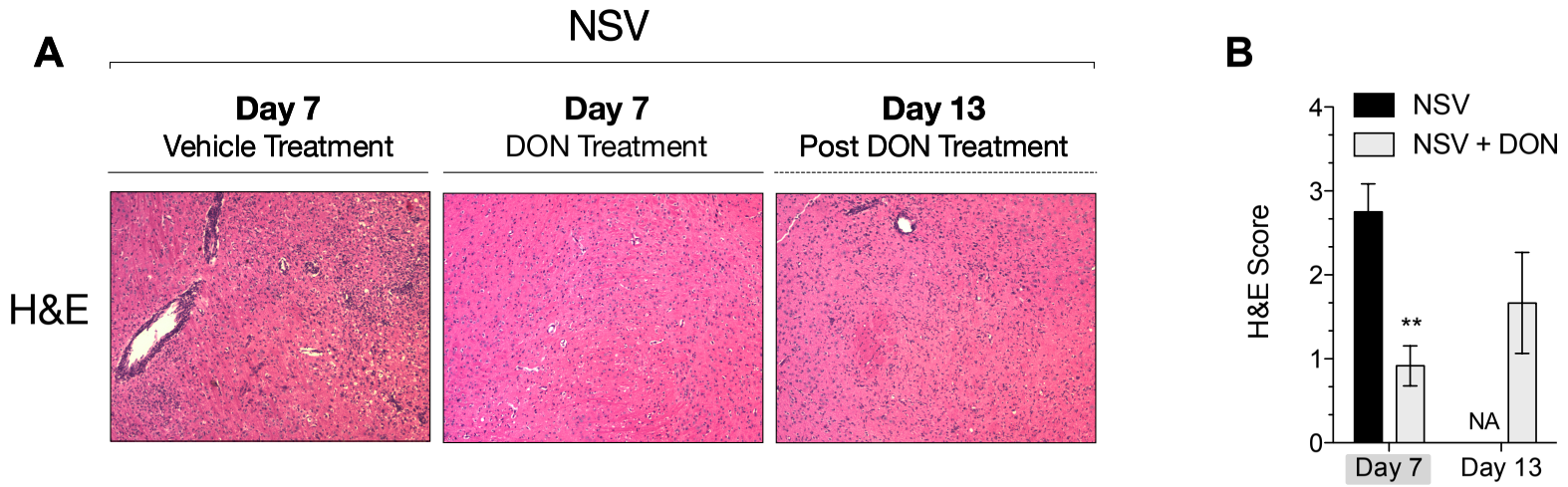


**Figure 2.5:** *Mononuclear cells appear after cessation of DON treatment in NSV-infected mice.* **(A)** Representative H&E-stained brain sections from day 7 (treated and untreated) and day 13 (after treatment). H&E scores quantified **(B)**. The H&E scoring criteria: (0) no inflammation, (1) one or two occasional inflammatory foci, (2) moderate foci in >50% 10x fields, (3) moderate-severe foci in >50% 10x fields. A score of +1 was given for abundant cellularity. Error bars represent +/-SEM of three mice per time point per group. Mice were treated with 0.3 mg/kg of DON or PBS once daily through Day 7. *P<0.05 (One-way ANOVA)

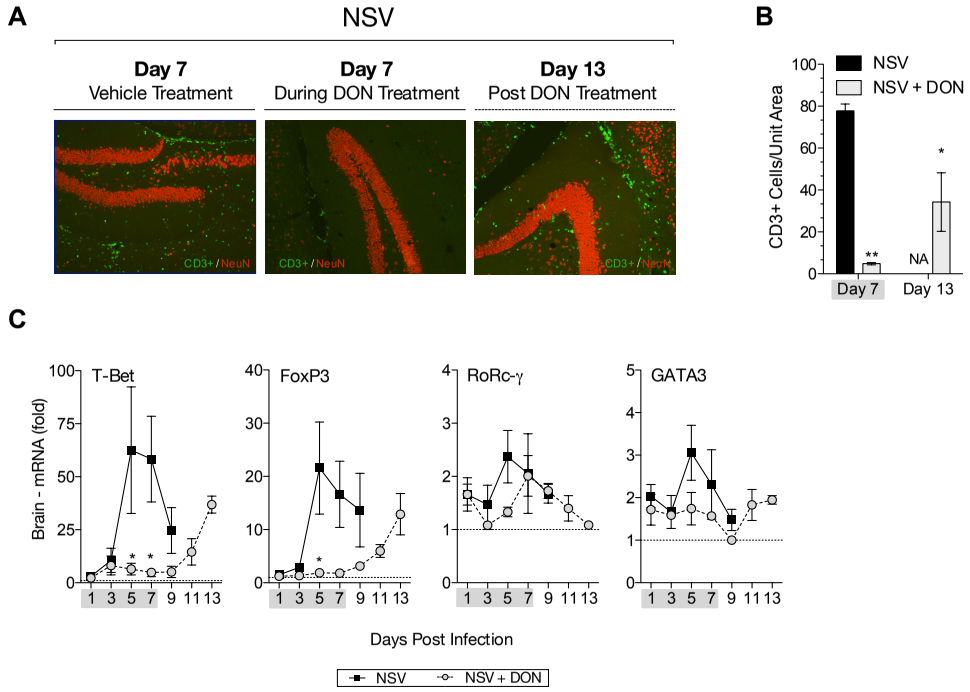


**Figure 2.6:** *The adaptive immune response appears after halting DON in treated mice.*
**(A)** Immunofluorescence of CD3+ lymphocytes (green) and NeuN, neuronal nuclei marker (red) in brain sections from day 7 (treated and untreated) and day 13 (after treatment) mice. CD3+ cells were quantified **(B)**. **(C)**mRNA expression of T-cell-specific transcription factors in the brain. Grey shading under the x-axis identifies the drug treatment period. Error bars represent +/-SEM of three mice per time point per group **(B,C)** Mice were treated with 0.3 mg/kg **(A,B)** or 0.6 mg/kg **(C)** of DON or PBS once daily through Day 7. *P<0.05; **P<0.01; ***P<0.001 (One-way ANOVA **(B)**, Two-way ANOVA with Bonferroni post test **(B)**).

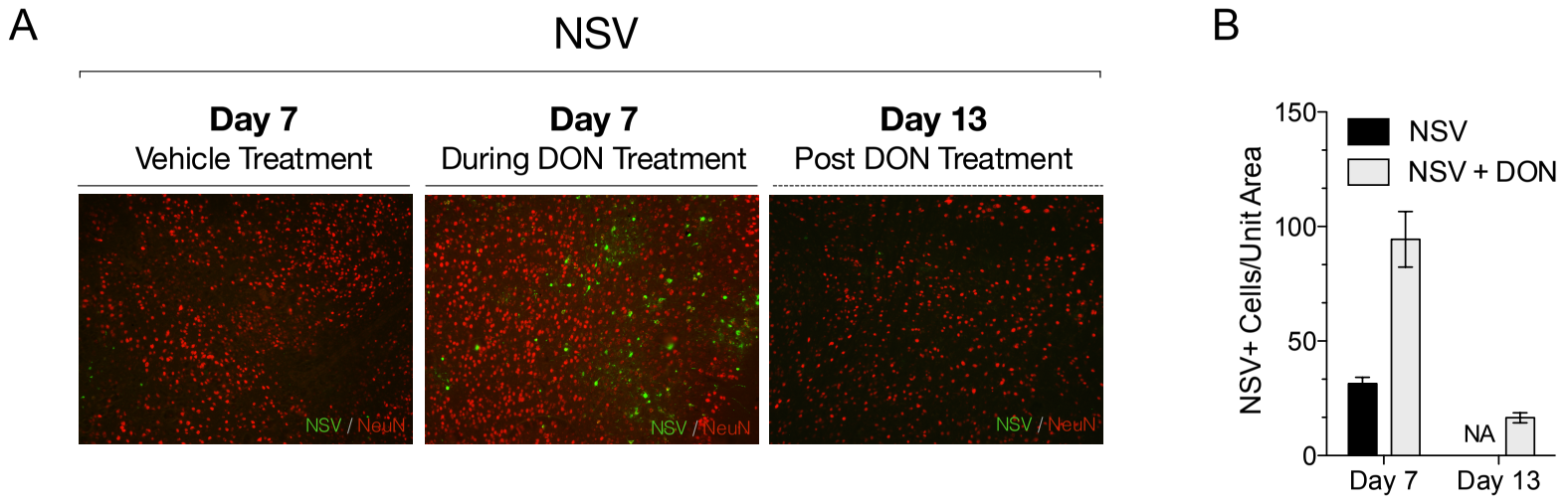


**Figure 2.7:** *NSV antigen is cleared after halting DON treatment.*
**(A)** Immunofluorescence of NSV positive neurons (green) and NeuN, neuronal nuclei marker (red) in brain sections from day 7 (treated and untreated) and day 13 (after treatement) mice. NSV positive neurons were quantified **(B)**.

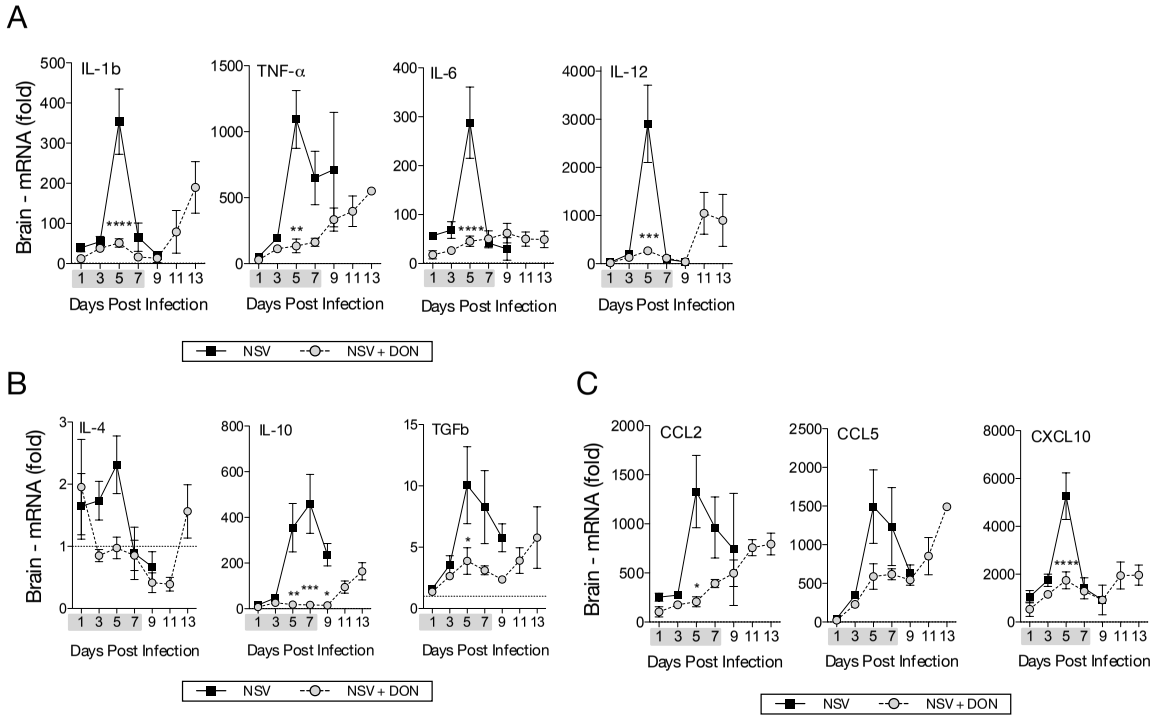


**Figure 2.8:** *Effect of treatment on the expression of cytokine and chemokine mRNAs in brain.* RNA from brains of treated (NSV + DON, 0.6mg/kg) and vehicle-treated (NSV) mice were examined for innate and adaptive cytokine and chemokine mRNAs (A) mRNA levels of innate proinflammatory cytokines (IL-1$\beta$, TNF-$\alpha$, IL-6, and IL-12). (B) mRNA levels of anti-inflammatory cytokines (IL-4, IL-10, and TGF-$\beta$) (C) mRNA levels of chemokines (CCL2, CCL5, and CXCL10). Grey bar under the x-axis represents the drug treatment period. Mice were treated with DON or PBS from Day 0 through Day 7. Error bars represent +/- SEM of three mice per time point. The dotted line in graphs (fold change equal 1) represent mRNA levels of the respective gene in uninfected mice. *P<0.05; ***P<0.001 (Two-way ANOVA with Bonferroni post test).

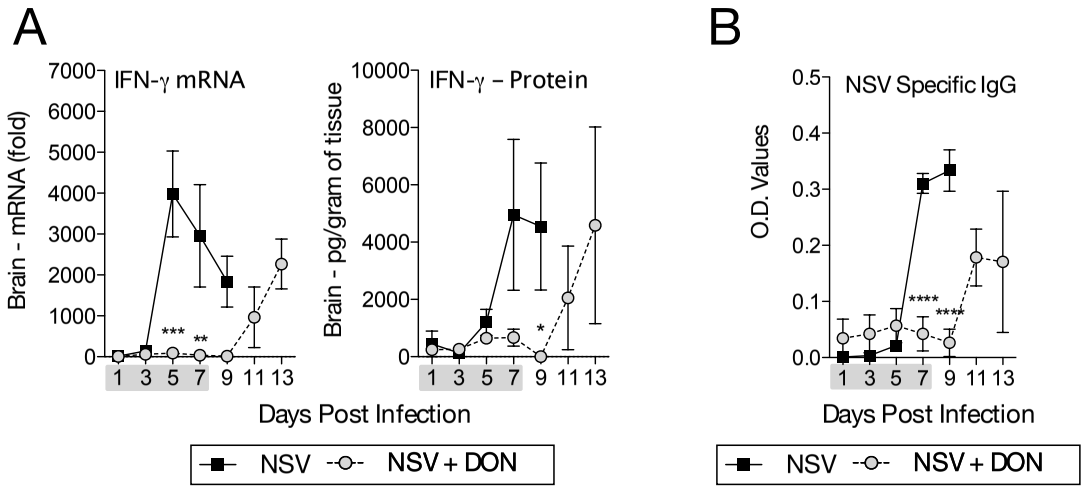


**Figure 2.9:** *DON-treated mice have less IFN-γ production in the brain and lower levels of NSV-specific antibody in the serum during treatment.*
**(A)** Brain IFN-γ mRNA levels as measured by qRT-PCR and normalized to GAPDH and IFN-γ protein levels measured by ELISA in brain homogenates from vehicle - (NSV) and DON-treated (NSV + DON, 0.6 mg/kg) mice. **(B)** Levels of NSV-specific IgG in serum measured by ELISA from DON-treated (NSV + DON) and vehicle-treated mice (NSV). Grey bar under the x-axis identifies the treatment period. Mice were treated with DON or PBS from day 0 through day 7. Error bars represent +/- SEM of three mice (N=3 mice/day/group). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 (Two-way ANOVA with Bonferroni post test).

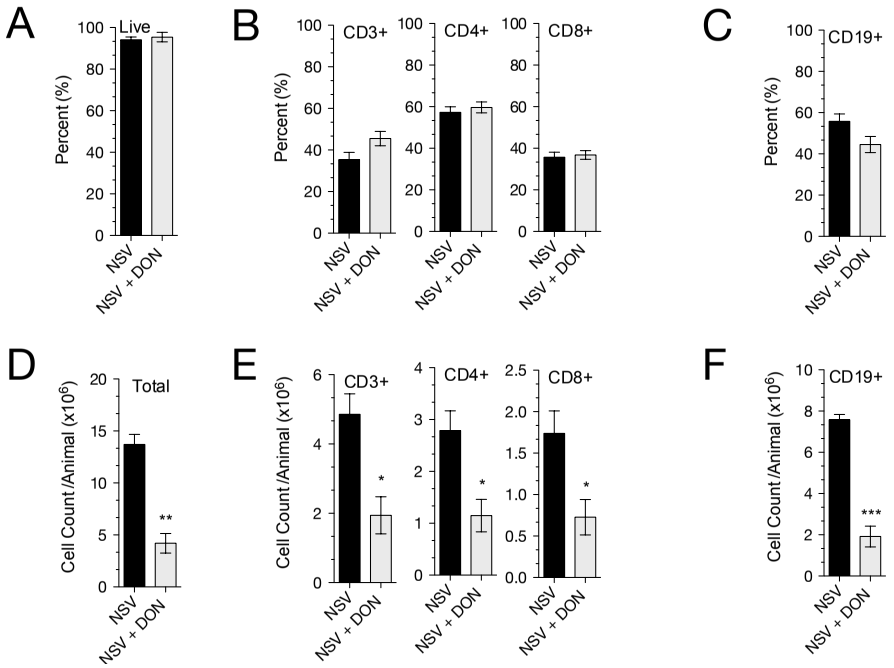


**Figure 2.10:** *Effect of DON treatment on lymphocyte counts in the superficial cervical lymph nodes.* Draining superficial cervical lymph nodes from NSV-infected treated (NSV + DON) and untreated (NSV) mice were harvested on Day 5, the peak proliferation period in NSV infected mice before the cells exit to the brain. Proportion of live cells **(A)**, CD3+, CD4+, CD8+ **(B)** and CD19+ **(C)** in the superficial draining cervical lymph nodes. Absolute counts of total cells **(D)**, CD3+, CD4+, CD8+ **(E)** and CD19+ **(F)** lymphocytes/animal in the superficial draining cervical lymph nodes. Mice were treated with 0.3 mg/kg of DON or PBS once daily through Day 7. Error bars represent +/- SEM of three mice per time point per group *P<0.05; **P<0.01; ***P<0.001; (Students t-test).

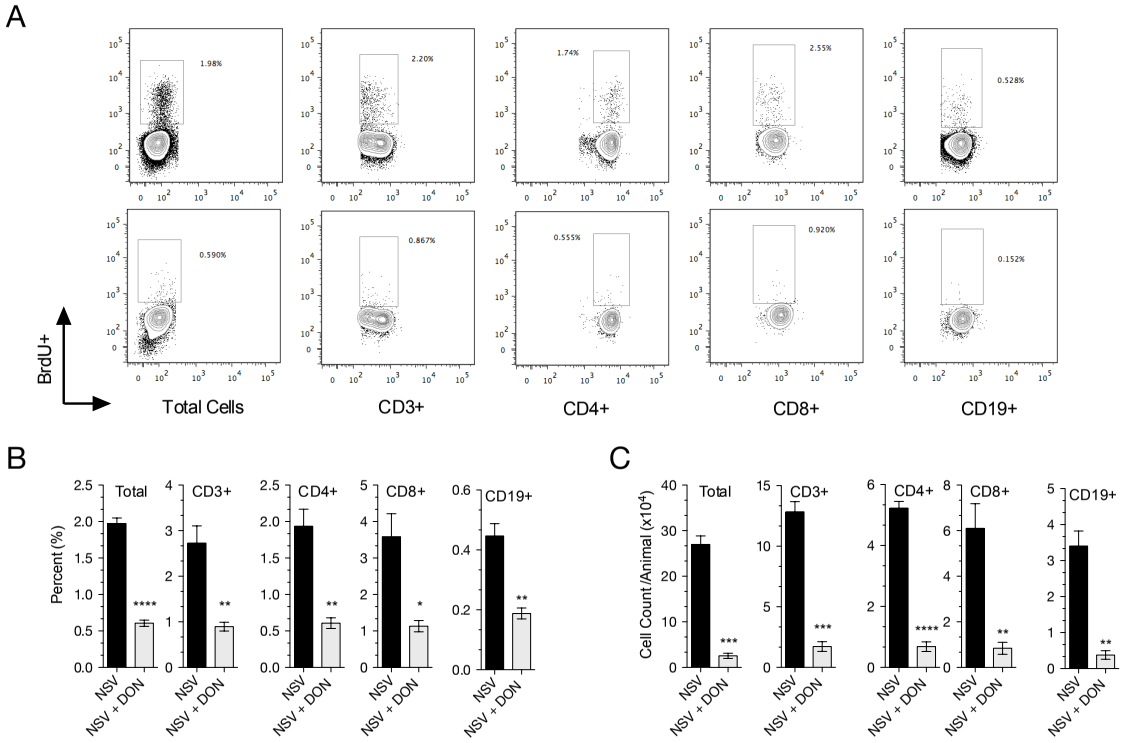


**Figure 2.11:** *Effect of DON treatment on peripheral lymphocyte proliferation in the superficial cervical lymph nodes.*

Mice were pulsed with BrdU to label proliferating cells on the days 4 and 5. Cells were collected 8h after pulsing and BrdU incorporation measured by flow cytometry and quantified by percent of cells incorporating BrdU **(A-B)** and the absolute number of BrdU+ cells **(C)**. Mice were treated with DON (0.3 mg/kg) or PBS from Day 0 through Day 7. Error bars represent +/- SEM of three mice per time point per group **(A)** or three mice **(B, C,D)**. Data are representative of two different independent experiments (B). *P<0.05; **P<0.01; ***P<0.001 (Two-way ANOVA with Bonferroni post test).

## 2.4 Discussion

Treatment for seven days with the glutamine antagonist DON protected mice from NSV-induced acute fatal paralysis. DON prevented induction of the anti-viral immune response, resulting in decreased CNS inflammation and a failure to clear infectious virus from the brain and spinal cord. Analysis of draining cervical lymph node cells showed that proliferation of B cells, and CD4+ and CD8+ T cells was prevented by DON treatment in infected mice. Mice that survived acute infection as a result of treatment succumbed to NSV-induced fatal encephalomyelitis after treatment was stopped, coincident with appearance of the anti-viral immune response, increased CNS inflammation and virus clearance.

Treatment with DON prevented the growth and proliferation of peripheral lymphocytes involved in the anti-viral immune response leading to decreased CNS lymphocyte infiltration and delayed viral clearance. Production of IFN-$\gamma$ and NSV-specific IgG, both important for virus clearance, were compromised in DON-treated mice. A role for the antiviral immune response in disease is empirically supported by the appearance of the pro-inflammatory, chemotactic, anti-inflammatory, and T-cell transcription factor mRNA expression in the brain after DON treatment was stopped. Previous studies have shown that susceptible C57BL/6 mice that are deficient in TCR-$\alpha/\beta$ in class I antigen presentation ($\beta$2 microglobulin, TAP1) or CD4+ T cells have improved survival after NSV infection and that IL-10-deficient mice have accelerated disease, suggesting that T-cells have a prominent role in NSV pathology [89,95,147]. However, neither the pharmacological depletion of circulating monocytes by clodronate-loaded liposomes nor the depletion of neutrophils alters the course of NSV-induced fatal disease [52,95].

These data suggest that T-cells are the primary mediators of neuronal damage in NSV-induced acute encephalitis. How T-cells mediate disease is still unknown, and prevention of disease development by DON may be through more than one mechanism. Previous studies have implicated glutamate excitotoxicity. Neurotropic infections of the CNS can lead to glutamate excitotoxicity both through direct neuronal damage and immune-mediated neuronal injury [7, 12, 13, 65, 125]. Cultured primary cortical and spinal cord neurons infected with NSV develop direct and bystander neuronal death via glutamate excitotoxicity, which can be ameliorated using antagonists of the NMDA and AMPA ionotropic glutamate receptors [39, 124]. In NSV-infected mice, treatment with NMDA and AMPA receptor antagonists protects hippocampal neurons against neurodegeneration, but only AMPA receptor antagonists protect spinal cord motor neurons and prevent NSV-induced fatal paralysis [65, 125]. Surprisingly, mice treated with AMPA receptor antagonists also showed delayed viral clearance, a decreased peripheral immune response and less CNS inflammation. Therefore, treatment with DON produced a similar outcome as treatment with AMPA receptor antagonists through a very different mechanism.

DON may prevent the generation of neurotoxic glutamate as well as inhibit the growth and proliferation of lymphocytes in peripheral lymphoid tissue. DON is effective in preventing the generation of glutamate from microglial cells and macrophages, and mitigation of disease in experimental autoimmune encephalomyelitis by DON is assumed to be due to prevention of this excitotoxic glutamate release [155]. However, as the current study has shown, DON also has potent inhibitory effects on induction of the immune response without affecting NSV replication in vitro or in vivo. Moreover, glutamine deprivation and treatment with drugs that inhibit glutamine metabolism

may have a direct effect on virus replication [31,56,64,76,80,129,144]. Although glutamine deprivation is reported to reduce titers of SINV produced by BHK cells [80], we were unable to replicate these effects, as neither glutamine deprivation nor DON treatment affected NSV viral titers in our studies.

However, DON is a relatively nonspecific inhibitor of all glutamine-utilizing enzymes and at high doses has been associated with substantial toxicity [49,159]. We have shown that acute treatment with low doses of DON is relatively well tolerated. GLS1, the major glutaminase involved in the generation of glutamate in the CNS [51], is also the glutaminase that is up regulated in T-cells during activation and has an central role in glutamine metabolism, cell cycle progression, and signaling in rapidly proliferating cells [23,33,47,62,97,169,185]. Because GLS1 plays a critical role in the nervous system as well as in the peripheral immune system, development of GLS1-specific inhibitors might offer a more specific, targeted and less toxic way to treat neuroinflammatory disease. Limiting neurotoxic glutamate production by neuronal and microglial cells in the CNS, as well as modulating the peripheral lymphocyte response, is a promising approach to treatment of viral encephalomyelitis. In future studies we plan to investigate the effect of specific glutaminase (GLS1) inhibitors on CNS glutamate excitotoxicity during NSV infection.

# CHAPTER 3

# EFFECTS OF GLUTAMINE ANTAGONISTS ON PRIMARY T-CELLS

## Contents



## Figures

## 3.1 Introduction

### 3.1.1 Glutamine metabolism in lymphocytes

T-cells preferentially use glutamine rather than glucose for metabolic needs during growth and proliferation [109]. Increased glutamine uptake and metabolism are characteristic of rapidly dividing cells such as activated T-cells and certain cancer cells where glutamine is used as an energy and nitrogen source [23]. Proliferating T-cells require millimolar concentrations of extracellular glutamine for growth and cell cycle progression [23]. T-cells stimulated in glutamine-deprived media fail to expand in size, replicate their DNA, or enter S-phase of the cell cycle [54, 118]. Additionally, silencing key enzymes involved in glutamine metabolism such as phosphate-activated glutamininase (GLS1), which is upregulated during T-cell activation, completely inhibits proliferation of human T-cells [33]. These studies suggest that availability and metabolism of glutamine is a critical metabolic bottleneck that can be pharmacologically exploited to prevent the expansion of activated T-cells, and modulate the immune response during neuroinflammatory diseases such as NSV-induced acute encephalomyelitis.

### 3.1.2 Glutaminase - GLS1

The enzyme glutaminase converts glutamine to glutamate and is widely expressed and highly regulated in the immune system [185]. T-cells express kidney-type glutaminase or GLS1, a distinct isoform expressed in non-hepatic tissues such as the brain, kidneys, immune system, and transformed cells [118]. Glutaminase mRNA and protein are up regulated early during T-cell activation when substantial amounts of glutamine are imported into T-cells [23, 75, 104, 137, 145, 185]. Human T-cells treated in vitro with either siRNA against GLS1 transcripts or treated with the competitive irreversible

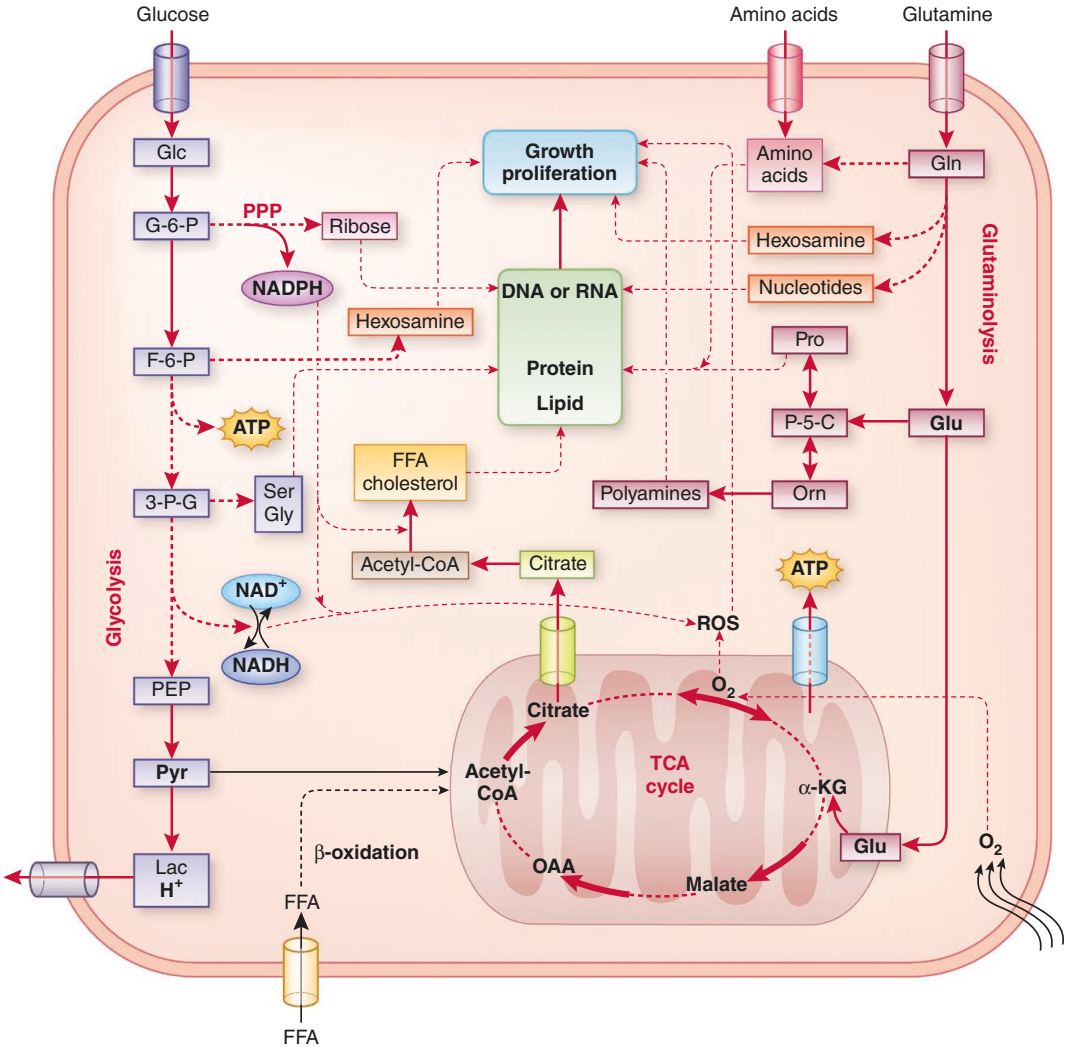


**Figure 3.1:** *Common metabolic pathways in T-cells*
Figures from: [186]

non-specific glutaminase inhibitor DON (6-diazo-5-oxo-L-norleucine), fail to initiate DNA synthesis and proliferate [33]. Moreover, other cells of the immune system, such as macrophages and neutrophils, show an increase in glutaminase activity upon activation [35–37, 195]. Additionally, cytokines such as IFN-$\alpha$, TNF-$\alpha$, and IL-1$\beta$ increase the expression of GLS1 [171, 191, 195].

### 3.1.3 T-cell activation

During induction of an immune response to infection, antigen-presenting cells (APC) in the local lymph node stimulate T-cells by engaging the antigen-specific T-cell receptor and costimulatory receptors. This process can be recapitulated in vitro by stimulating T-cells with antibodies to CD3 (T cell receptor) and CD28 (costimulatory receptor). Cross-linking of CD3 and CD28 stimulates a series of proximal signaling events that activate the MAPK, NFAT, and NF-B signaling pathways. These transcription factors induce the production of the T cell growth factor interleukin (IL)-2 and expression of the high affinity receptor CD25/IL-2R. IL-2 is released into the extracellular space and engages the IL-2R on the T-cell plasma membrane in an autocrine and paracrine manner to induce T-cell proliferation. Downstream signaling from the IL-2R and co-stimulatory receptors activate genes and pathways necessary for cellular growth and proliferation, the central controller of which is the mTOR pathway. The mTOR pathway, specifically mTORC1, in T-cells integrates nutrient and mitogen signals to facilitate translation of essential proteins such as growth factors [109, 117, 119]. Currently used immunosuppressive drugs work either by blocking transcription of the IL-2 gene (i.e. cyclosporin and FK506) or blocking the downstream effects of IL-2 on T-cells (i.e. rapamycin).

Expression of early activation markers CD69 and CD25 are dependent on the

initial activation cascade of NFAT, MAPK and NF-kB which induces transcription of the IL-2 gene. Hence, surface expression of CD69 and CD25 markers 6-24 hours after anti-CD3/anti-CD28 stimulation suggests a functional activation cascade. CD25 is kept on the surface with a IL-2 autocrine and paracrine feedback loop. Moreover, upregulation of CD98, an amino acid transporter, and CD71 (transferrin receptor) occurs during late activation.

### 3.1.4 How does glutamine antagonism inhibit T-cell proliferation?

In the previous chapter we showed that DON had a direct effect on induction of the peripheral immune response. DON-treated mice had less lymphocyte proliferation in the peripheral lymph nodes, decreased CNS lymphocyte infiltration, and delayed viral clearance compared to untreated mice. In this chapter, we explore the direct effect of glutamine antagonism on lymphocyte proliferation. We show that glutamine deprivation and treatment with glutamine antagonists, DON and ACI, directly inhibit lymphocyte growth, proliferation, and cytokine production.

## 3.2 Material and Methods

### 3.2.1 Cell Culture and Drugs

Primary lymphocytes were grown in DMEM supplemented with 10% dialyzed FBS (Invitrogen), penicillin (pen), streptomycin (strep), 2mM glutamine, and 50$\mu$M $\beta$-mercaptoethanol (Sigma). Glutamine-deficient DMEM was used in certain experiments. All cells were grown at 37°C with 5% $CO_2$. Glutamine antagonists, 6-diazo-5-oxo-L-norleucine (DON; Sigma) and Acivicin (ACI; Sigma) were solubilized in sterile PBS to prepare 100mM stock solutions. Working dilutions were made in media for in vitro experiments or in sterile PBS for in vivo experiments. Stock solutions were

stored at -80℃ and fresh working solutions were created for each use.

### 3.2.2 In vitro T-cell analysis

CD3+ lymphocytes were purified from the spleens of adult C57BL/6J mice using a Pan T-cell Isolation Kit (Miltenyi Biotec). For activation, flat bottom 96-well plates were coated with anti-CD3 (5 $\mu$g/mL, 145-2C11 clone, eBiosciences) and anti-CD28 (2.5 $\mu$g/mL, 37.51 clone, eBiosciences) for 2h at 37℃ and washed. Prior to culture, lymphocytes were stained with CFSE (Invitrogen) in PBS with 0.1% BSA for 5 min at 37℃, washed, and then added at a density of $10^5$ cells/well. Cells were cultured in glutamine-free or complete (DMEM, 10% dialyzed FBS, 2mM glutamine, NEAAs, 25mM HEPES) media in the presence or absence of DON or ACI 5-20$\mu$M. Cells were stained for CD3, CD4 (PerCPCy 5.5, RM4-5 clone, BD Biosciences), and CD8 as described above. Viability was assessed at 12h by flow cytometry (BD FACS Canto II) using a violet fluorescent exclusion dye (Invitrogen) according to the manufacturers protocol. T-cell proliferation was assessed at 72h by CFSE dilution.

### 3.2.3 Enzyme immunoassays

For measurement of IFN-$\gamma$ and IL-2, supernatant fluids collected from culture media 24h after activation of CD3+ splenocytes with anti-CD3 and CD28 were assayed for IFN-$\gamma$ and IL-2 by ELISA(R&D Systems) according to the manufacturer's protocol. Briefly, 96-well plates were coated overnight at 4℃ with capture antibody. Plates were washed with PBST (PBS with 0.05% Tween-20), blocked with 1% BSA in PBST for 1h at RT or overnight at 4℃ and washed. Plates were then incubated with detection antibody for 2 hours, washed and subsequently incubated with Streptavidin-HRP. Color was developed with TMB (3,3,5,5-tetramethylbenzidine) substrate solution (Sigma). After adding stop solution (2M $H_2SO_4$), optical densities (ODs) at

450nm were determined.

## 3.3 Results

### 3.3.1 DON treated T-cell failed to proliferate in vitro.

To determine the effects of glutamine deprivation on T-cell proliferation in vitro, we stimulated purified primary splenic T-cells with anti-CD3/anti-CD28 and measured growth and proliferation. Treatment with glutamine antagonists DON or ACI did not affect cell viability (Supplemental Figure B.2), but drug-treated cells failed to proliferate. CFSE dilution profiles showed that, like glutamine-deprived lymphocytes, DON and ACI-treated lymphocytes failed to grow in size (Figure 3.2) and failed to undergo even one round of division after stimulation (Figure 3.3A). The profiles of DON and ACI-treated stimulated lymphocytes were similar to glutamine-deprived stimulated lymphocytes and to unstimulated lymphocytes (Figure 3.3A, B). Because production of IL-2 is essential for lymphocyte growth in response to stimulation, IL-2 was measured. Glutamine-deprived and DON and ACI-treated lymphocytes did not produce IL-2 (Figure 3.3C) or IFN-$\gamma$ (Figure 3.3D) in response to stimulation. We found similar results using a glutaminase (GLS1) inhibitor, JHU-212 (Supplementary Figure B.3 ). Additionally, treatment with downstream metabolite, $\alpha$-ketoglutarate, failed to fully rescue proliferation in JHU-212 treated lymphocytes (Appendix Figure B.5). Mice partially deficient in global GLS1 levels showed no difference in IL-2 production or proliferation (Appendix Figure B.4).

### 3.3.2 DON-treated T-cells failed to fully activate upon stimulation

To determine the effects of glutamine deprivation on T-cell activation, early activation markers CD69 and CD25 were examined 24h post stimulation. Treatment with

glutamine antagonists and glutamine deprivation moderately inhibited CD69 expression while CD25 expression was more severely compromised (Figure 3.4). Growth and proliferation markers CD98 and CD71 were also analyzed by flow cytometry 24 hours post stimulation (Figure 3.5). T-cells in glutamine-deprived media as well as cells treated with glutamine antagonists showed a severe defect in the expression of CD71 which is controlled by the mTOR1 pathway [196]. Therefore we examined the phosphorylation of ribosomal protein S6 downstream in the mTOR pathway. We found that DON and JHU-212 inhibited S6 phosphorylation (Appendix Figure B.6), similar to effect of glutamine deprivation found by others [185].

## 3.4 Discussion

Glutamine metabolism is important in neuroinflammatory diseases. Glutamine is utilized by the CNS as a source of the neurotransmitter glutamate and in peripheral lymphoid tissue as an essential metabolite for growth and proliferation of lymphocytes. In this chapter, in vitro experiments using primary mouse T-cells were used to confirm the immunosuppressive effects of DON. DON-treated CD3+ lymphocytes failed to grow and proliferate.

The upregulation and regulation of glutamine metabolism is controlled by the transcription factor Myc which is increased during late stages of T-cell activation [142, 185]. There has been a recent resurgence of interest in metabolism of immune cells, in particular T-cells. Recent studies have shown that different T-cell subsets have different metabolic programs [109]. Activated T-cells upregulate both glutamine and glucose metabolism with a preference towards glutamine utilization for rapid expansion [23,54]. Effector cells have robust glycolytic metabolism while naive, memory,

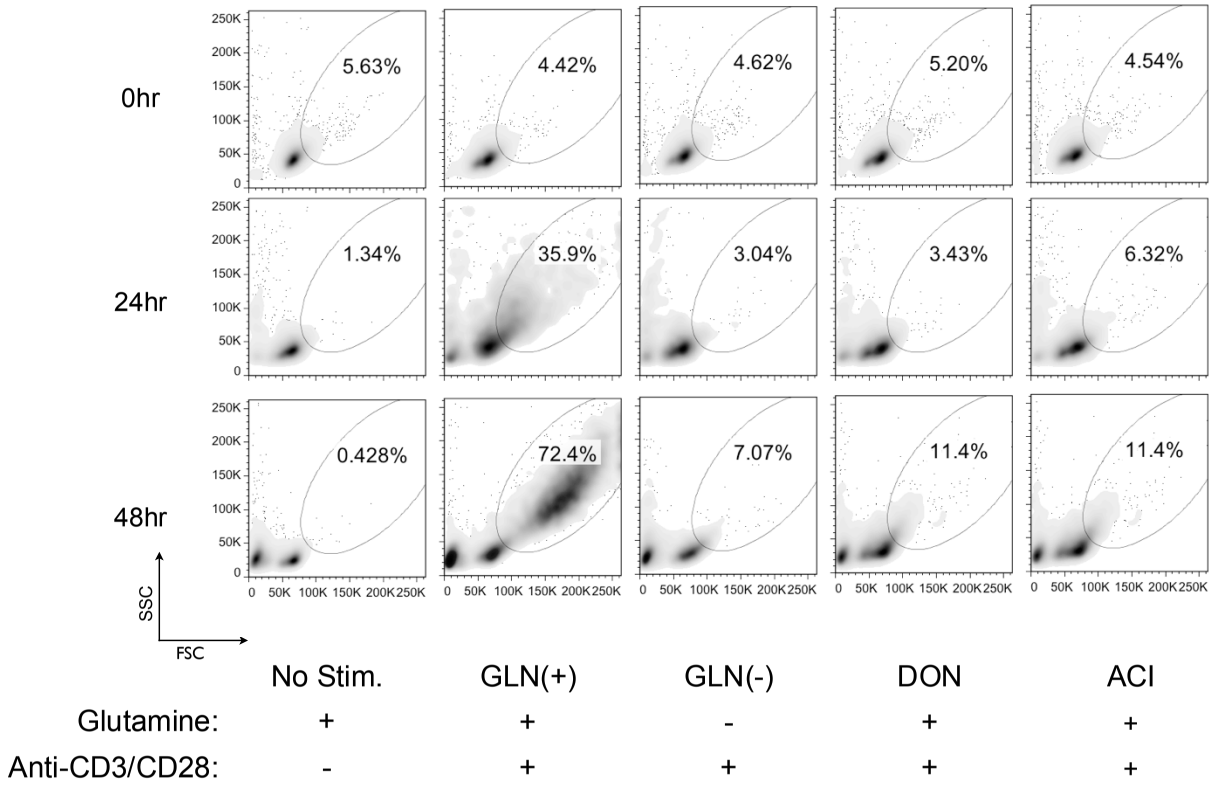


**Figure 3.2:** *DON-treatment inhibits T-cell growth.*
CD3+ T-cells isolated from mouse spleens were stimulated with anti-CD3/anti-CD28 for 0, 24, or 48h. Stimulated cells were either supplemented with glutamine (Gln(+), deprived of glutamine (Gln(-)), or treated with glutamine antagonists (DON and ACI ) in glutamine-supplemented media. Growth in size (FSC) and complexity (SSC) was measured by flow cytometry.

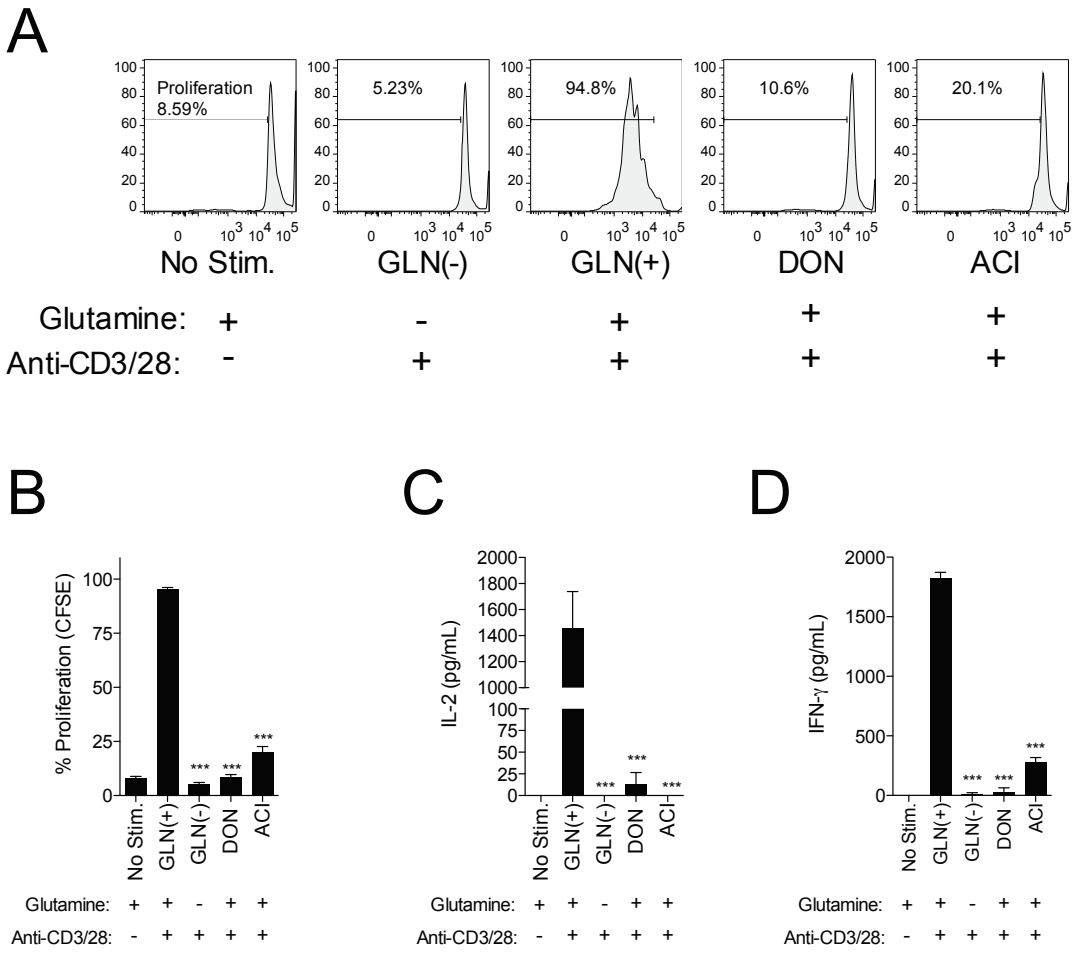


**Figure 3.3:** *DON treated T-cells fail to proliferate and produce IL-2 and IFN-$\gamma$* Spleen CD3+ T-cells were stimulated with anti-CD3/anti-CD28. Stimulated cells were either supplemented with 2mM glutamine (Gln(+), deprived of glutamine (Gln(-)), or treated with glutamine antagonists (DON and ACI ) in glutamine-supplemented media. **(A)** CFSE proliferation assay of stimulated T-cells after 72 h. **(B)** Quantitation of CFSE data. ELISA analysis of the T-cell growth factor IL-2 **(C)** and IFN-$\gamma$ (D). (Error bars represent +/- SEM of mean of three biological replicates. Data are representative of at least two independent experiments. ***P <0.001 (One-way ANOVA with Dunett's post test)

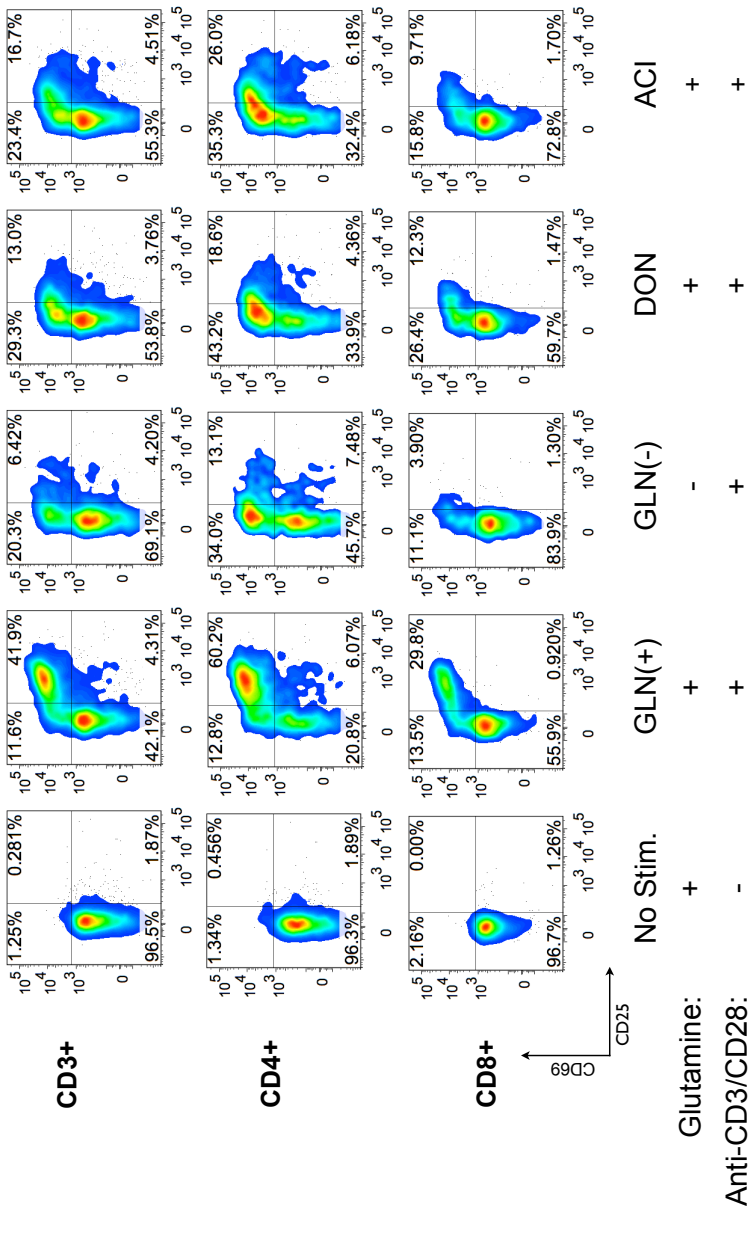


**Figure 3.4:** *Effect of glutamine deprivation and antagonists on CD69 and CD25 expression.*
Spleen CD3+ T-cells were stimulated with anti-CD3/anti-CD28 for 24h and analyzed by flow cytometry for presence of CD69 and CD25 activation markers. Stimulated cells were either supplemented with glutamine (Gln(+), deprived of glutamine (Gln(-)), or treated with glutamine antagonists (DON and ACI ) in glutamine-supplemented media.

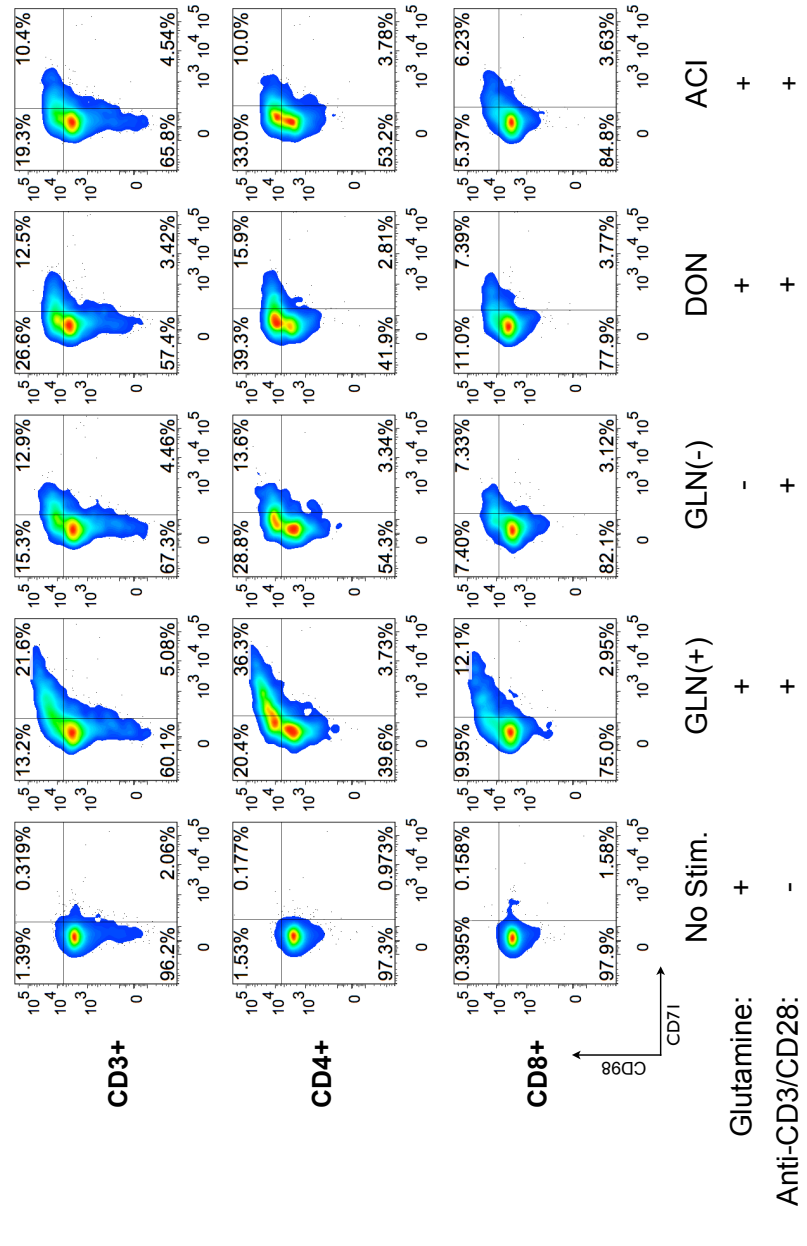


**Figure 3.5:** *Effect of glutamine deprivation and antagonists on CD98 and CD71 expression.*
Spleen CD3+ T-cells were stimulated with anti-CD3/anti-CD28 for 24h and analyzed by flow cytometry for presence of CD98 and CD71 growth and proliferation markers. Stimulated cells were either supplemented with glutamine (Gln(+), deprived of glutamine (Gln(-)), or treated with glutamine antagonists (DON and ACI ) in glutamine-supplemented media.

T-regulatory cells survive on fatty acid metabolism [109]. In proliferating cells, glutamine contributes to replenishment of the TCA intermediate $\alpha$-ketoglutarate and to the *de novo* synthesis of nucleotides required for DNA replication during the S-phase of the cell cycle [54]. The availability of glutamine is an important metabolic checkpoint. Cells deprived of glutamine are arrested before reaching the S-Phase of the cell cycle [54, 185]. In the previous chapter we showed that T-cells of NSV-infected and DON-treated mice failed to incorporate the thymidine analogue, BrdU (Figure 2.11), suggesting an arrest in the transition from G1 to S-phase of the cell cycle. We replicated previous findings by others that showed that activated lymphocytes deprived of extracellular glutamine fail to proliferate or produce cytokines such as IL-2 [23, 109]. In addition, we demonstrated that effects of glutamine deprivation can be replicated using glutamine antagonists DON and ACI. Importantly, unlike in cancer cells, treating primary T-cells with downstream anaplerotic metabolites like $\alpha$-ketoglutarate cannot rescue depreviation of glutamine availability and metabolism in primary lymphocytes [185]. We showed that inhibition of proliferation by treatment with the glutaminase (GLS1) inhibitor JHU-212 cannot be rescued by supplementation with $\alpha$-ketoglutarate (Appendix Figure B.5) [152]. However, others have shown limited rescue of proliferation is possible through the use of nucleotide precursors [185].

The precise mechanism by which cells are able to sense the availability of glutamine is an area under intense investigation. Recent studies have shown that upstream glutamine is transport channels and the mTOR pathway play an important role in sensing the presence of glutamine in cells [47, 123, 128]. Moreover, glutaminolysis mediated by GLS1 plays key roles in activating the mTOR pathway [47]. Nutrient sensing via the mTOR pathway may be pivotal role in the late stage of activation and proliferation of lymphocytes [47, 117, 123, 128, 182]. Glutamine deprivation limits

the phosphorylation of the ribosomal protein S6, a downstream mTOR target, in T-cells [185]. We showed that treatment with glutamine antagonists, DON, and GLS1 inhibitor JHU-212 resulted in a similar defect in phospho-S6 levels (Figure B.6) suggesting, that DON and JHU-212 affect the mTOR pathway. Others, have also suggested that the MAPK pathway could also play a role [23] and it should be noted that there can be significant cross talk between the Erk and mTOR pathways [115].

Antigen-specific T-cells must rapidly expand to control invading pathogens. Unlike proliferating cancer cells that can have heterogeneous metabolism, proliferating primary T-cells are severely restricted by the availability of glutamine. This rapid expansion puts extreme metabolic demands on antigen-specific T-cells. Consistent with other studies we showed a defect in the ability of lymphocytes to proliferate and produce cytokines under glutamine deprivation. In addition, we showed that the effects of glutamine deprivation can be achieved by the use of glutamine antagonists. Therapeutically modulating glutamine metabolism in primary T-cells might be important in controlling inflammatory disease.

# CHAPTER 4

# EFFECTS OF AMPA RECEPTOR ANTAGONISTS ON ACTIVATION OF PRIMARY T-CELLS.

## Contents



## Figures

## 4.1 Introduction

### 4.1.1 AMPA Receptors

A neuroadapted strain (NSV) of Sindbis virus, the prototypic alphavirus causes fatal encephalomyelitis and paralysis in weanling mice. Previous studies using this model have shown an interesting non-canonical role for AMPA receptor (AMPAR) antagonists on lymphocyte proliferation that has implications for the treatment of CNS and non-CNS immune-mediated diseases.

AMPA receptors are glutamate-gated ion channels canonically found on mammalian neurons for excitatory neurotransmission. AMPA receptors are composed of a tetrameric combination of subunits, GluR1-4, in the form of a homo or hetero dimers. The distribution and composition of AMPA receptors in the CNS is heterogenous. The presence of a post-transcriptionally edited GluR2 subunit confers Ca++ impermeability to the ion channel. GluR2 is post-transcriptionally edited with close to a 100% efficiency in the brain. Hence, GluR2 containing AMPA receptors are impermeable to calcium in the brain.

Because of the interest in use of neuroprotective agents, such as AMPAR antagonists for prevention of CNS injury, development of drugs that modify AMPAR signaling is an active area of research [55, 161, 183]. Both competitive and non-competitive AMPAR antagonists have been developed. The competitive antagonists have shown problems with in vivo toxicity and are not currently being developed for clinical use. The prototype non-competitive antagonists are 2,3 benzodiazepine derivatives. The first to be developed was 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3 benzodiazepine (GYKI-52466) [45]. The later generation

(R)-7-acetyl-5-(4-aminophenyl)-8,9-dihydro-8-methyl-7H-1,3-dioxolo(4,5-h)(2,3) benzodiazepine (GYKI 53773/LY300164/talampanel) is orally available, protects neurons from excitotoxic damage in animal models of brain trauma, ischemia, and epilepsy [156, 161].

### 4.1.2 AMPA Receptor antagonists protect from NSV-induced fatal disease.

GYKI-52466 and Talampanel belong to a class of 2,3 benzodiazepines being developed to prevent glutamate excitotoxicity in the central nervous system (CNS) [45]. These drugs allosterically inhibit non-NMDA glutamate-gated ion channels present on neurons. In particular, 2,3 benzodiazepines are highly selective for AMPA receptors over NMDA and kainate receptors. Previous in vivo studies in our lab have shown that mice treated with GYKI-52466, or its more potent analogue, Talampanel are protected from fatal NSV-induced encephalomyelitis and paralysis [65, 125]. NMDA antagonist, MK-801, does not protect these mice from death and paralysis [125]. Treatment did not affect virus distribution or maximum virus titers, but did dramatically reduce glial cell activation and inhibited the induction of an antiviral immune response and CNS inflammation suggesting that this class of drugs might also have a direct effect on neuroinflammation in addition to preventing glutamate excitotoxicity [65, 125].

### 4.1.3 Do AMPA receptor antagonists have a direct effect on lymphocyte proliferation?

Both ionotrophic AMPA and NMDA glutamate receptors are present on peripheral lymphocytes [57]. However, little is known about the impact of AMPA receptor antagonists on lymphocyte function. Using primary T-cells we show that AMPA receptor antagonists have direct effects on lymphocyte activation. Primary lymphocytes

treated with GYKI fail to proliferate, produce IL-2, express early activation marker CD69, and have defects in sustained Erk activation. Demonstration that AMPA receptor antagonists can have a direct effect on the activation of primary T-lymphocyte has wide implications for the use of AMPA receptor antagonsits for treatment of neuroinflammatory diseases.

## 4.2 Material and Methods

### 4.2.1 Cell Culture and Drugs

Primary lymphocytes were grown in DMEM supplemented with 10% dialyzed FBS (Invitrogen), non-essential amino acids (NEAAs w/o glutamine), penicillin (pen), streptomycin (strep), 2mM glutamine, and 50$\mu$M $\beta$-mercaptoethanol (Sigma). Glutamine-deficient DMEM was used in certain experiments. EL4.IL2 cells were cultured in DMEM supplemented with 10% FBS, pen/strep, and 2mM glutamine. All cells were grown at 37℃ with 5% CO2.

AMPA receptor antagonist, GYKI-52466 (Sigma) was dissolved in 10% DMSO in 0.1M HCl at a concentration of 26mM. GYKI-53655 was solubilized in sterile DMSO at a 26mM or 100mM stock concentration. Working dilutions were made in media for in vitro experiments. Ionomycin (1mg/mL) and PMA (Phorbol 12-myristate 13-acetate) were dissolved in DMSO. Stock solutions were stored at -20℃ and fresh working solutions were created for each use.

### 4.2.2 In vitro T-cell analysis

CD3+ lymphocytes were purified from the spleens of adult C57BL/6J (Jackson Labs) or CD-1 (Charles River) mice using a Pan T-cell Isolation Kit (Miltenyi Biotec). For

activation, flat bottom 96-well plates were coated with anti-CD3 (5 $\mu$g/mL, 145-2C11 clone, eBiosciences) and anti-CD28 (2.5 $\mu$g/mL, 37.51 clone, eBiosciences) for 2h at 37℃ and washed. Prior to culture, lymphocytes were stained with CFSE (Invitrogen) in PBS with 0.1% BSA for 5 min at 37℃, washed, and then added at a density of 1-3x$10^5$ cells/well. Cells were cultured in glutamine-free or complete (DMEM, 10% dialyzed FBS, 2mM glutamine, NEAAs, 25mM HEPES) media in the presence or absence of GYKI-52466 150$\mu$M or GYKI-53655 5$\mu$M. Cells were stained for CD3, CD4, and CD8 (BD Biosciences)) as described above. Viability was assessed at 12h by flow cytometry (BD FACS Canto II) using a violet fluorescent exclusion dye (Invitrogen) according to the manufacturers protocol. T-cell proliferation was assessed at 72h by CFSE dilution.

### 4.2.3 Enzyme immunoassays

For measurement of IL-2, supernatant fluids collected from culture media 12-24h after activation of CD3+ splenocytes with anti-CD3 and anti-CD28 were assayed for IL-2 (R&D Systems). According to the manufacturer's protocol. Briefly, 96-well plates were coated overnight at 4℃ with capture antibody. Plates were washed with PBST (PBS with 0.05% Tween-20), blocked with 1% BSA in PBST for 1h at RT or overnight at 4℃ and washed. Plates were then incubated with detection antibody for 2 hours then washed and subsequently incubated with Streptavidin-HRP. Color was developed with TMB (3,3,5,5-tetramethylbenzidine) substrate solution (Sigma). After adding stop solution (2M $H_2SO_4$), optical densities (ODs) at 450nm were determined.

## 4.3 Results

### 4.3.1 GYKI-53655 inhibits proliferation of primary T-cells

CD3+ lymphocytes from the spleens of C57BL/6 mice were activated in 96-well plates coated with anti-CD3/anti-CD28 and treated with GYKI-53655 at half-log dose intervals or vehicle (DMSO). Cells treated with GYKI at concentrations greater than 1$\mu$M failed to grow in size and complexity (Figure 4.1A, top panel) and proliferate (Figure 4.1A, bottom panel, B) in response to stimulation compared to vehicle control. At the higher doses of GYKI stimulated lymphocytes failed to undergo multiple rounds of proliferation as indicated by their inability to dilute CFSE. At 3$\mu$M and 10$\mu$M primary T-cells failed to undergo even one round of replication. IL-2 is an important T-cell growth factor, essential for lymphocyte growth in response to stimulation. IL-2 was measured 24 hours post stimulation. GYKI-treated T-cells failed to produce IL-2 as measured by ELISA. GYKI-52466, the prototypic 2,3 benzodiazepine had the same effect on lymphocyte proliferation and IL-2 production at higher concentrations (Figure C.1, 4.2) Supplementing the media with IL-2 failed to rescue lymphocyte proliferation (Figure 4.2C).

### 4.3.2 GYKI-53655 inhibits T-cell activation.

To determine if GYKI inhibited T-cell activation. We looked at the early activation markers CD69 and CD25 in drug-treated and untreated T-cells by flow cytometry. While CD25 expression was only marginally affected, only half of GYKI treated cells expressed CD69 (Figure 4.3). GYKI did not affect cell viability compared to vehicle-treated controls (Figure 4.3A). Previous studies have shown that CD69 is regulated via the Ras-Erk pathway which is inducible by PMA (Phorbol Myristate Acetate), a diacylglycerol DAG agonist [38,165]. Treating lymphocytes with PMA alone induced

CD69 surface expression (Figure 4.4). PMA-induced CD69 expression was inhibited by drug treatment (Figure 4.4).

### 4.3.3 GYKI-53655 inhibits sustained Erk activation in EL4.IL2 cells

EL4.IL2 cells are a mouse lymphoblast cell line capable of producing IL-2 after stimulation with PMA or plate bound anti-CD3/anti-CD28 [59, 72, 154]. IL-2 production after PMA (Appendix Figure 4.5) or anti-CD3/anti-CD28 (Figure 4.6A) stimulation was inhibited in drug-treated cells compared to vehicle controls. To determine the effect of drug treatment on activation of downstream kinases of the Ras pathway, we probed the phosphorylation status of Jnk, p38, and Erk by western blot. There were no differences in phosphorylation of Jnk or p38 between drug and vehicle-treated cells. However, GYKI-treated EL4.IL2 cells showed a defect in sustained Erk phosphorylation compared to vehicle-treated cells (Figure 4.6B).

## 4.4 Discussion

Glutamate is the primary excitatory neurotransmitter in the CNS. Mice treated with AMPA, but not NMDA, receptor antagonists are protected from NSV-induced acute fatal encephalomyelitis [65, 125]. In these studies, AMPA receptor antagonists protected mice by inhibiting the induction of the peripheral anti-viral immune response resulting in delayed viral clearance. In this chapter we examined the direct affect of AMPA receptor antagonists on CD3+ lymphocyte proliferation.

Primary mouse lymphocytes treated with GYKI-52466 (Figure C.1, 4.2) or GYKI-53655 failed to produce the essential lymphocyte growth factor IL-2 after stimulation

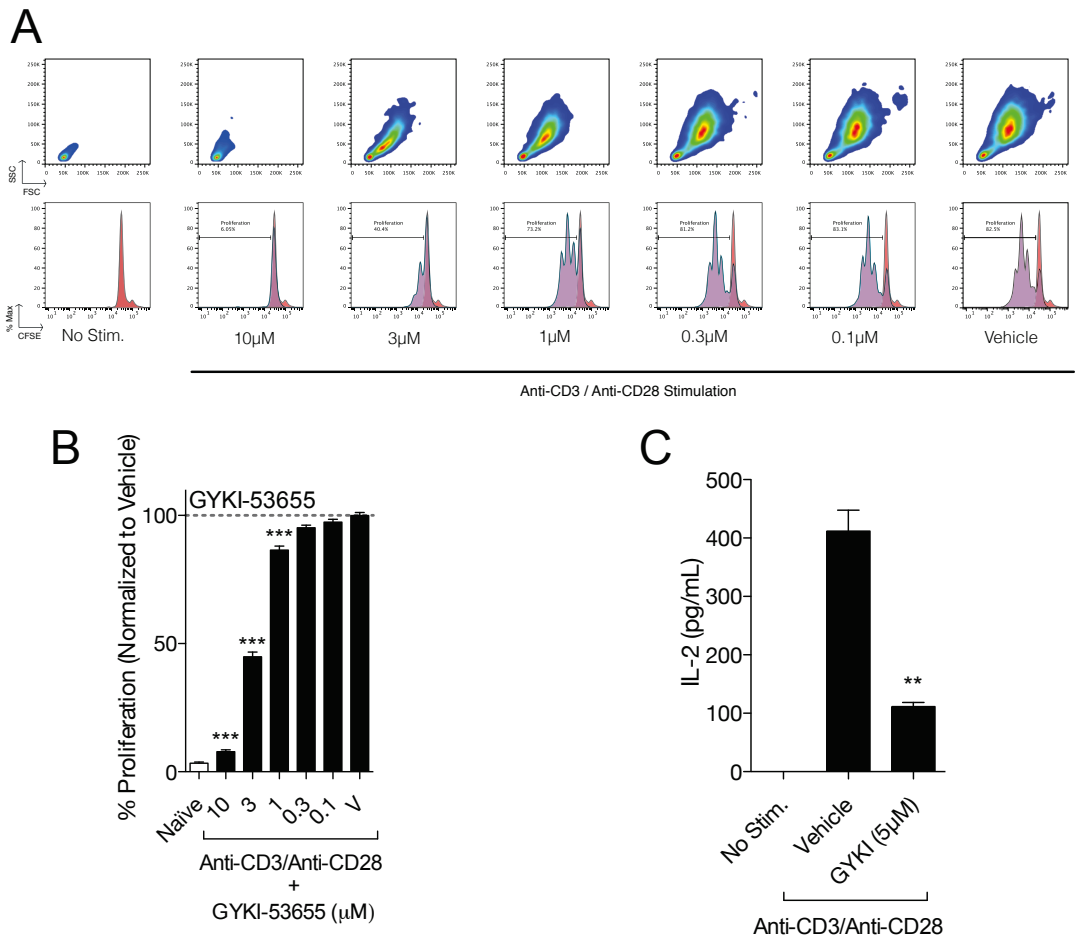


**Figure 4.1:** *GYKI-53655, inhibits T-cell proliferation in vitro*
CD3+ T-cells isolated from mouse spleens were stimulated with anti-CD3/anti-CD28 for 72h. Growth in size (FSC) and complexity (SSC) was measured by flow cytometry (top panel, **(A)**. Proliferation measured by CFSE dilution assay (bottom panel, **(A)**) and quantified **(B)**. IL-2 levels of primary CD3+ lymphocytes treated with either GYKI-53655 (5$\mu$M) or Vehicle 24 hours after activation **(C)**. Error bars represent +/- SEM of mean of three biological replicates. **P <0.01; ***P <0.001 (One-way ANOVA with Dunett's post test **(B)** or Student's t-test **(C)**)

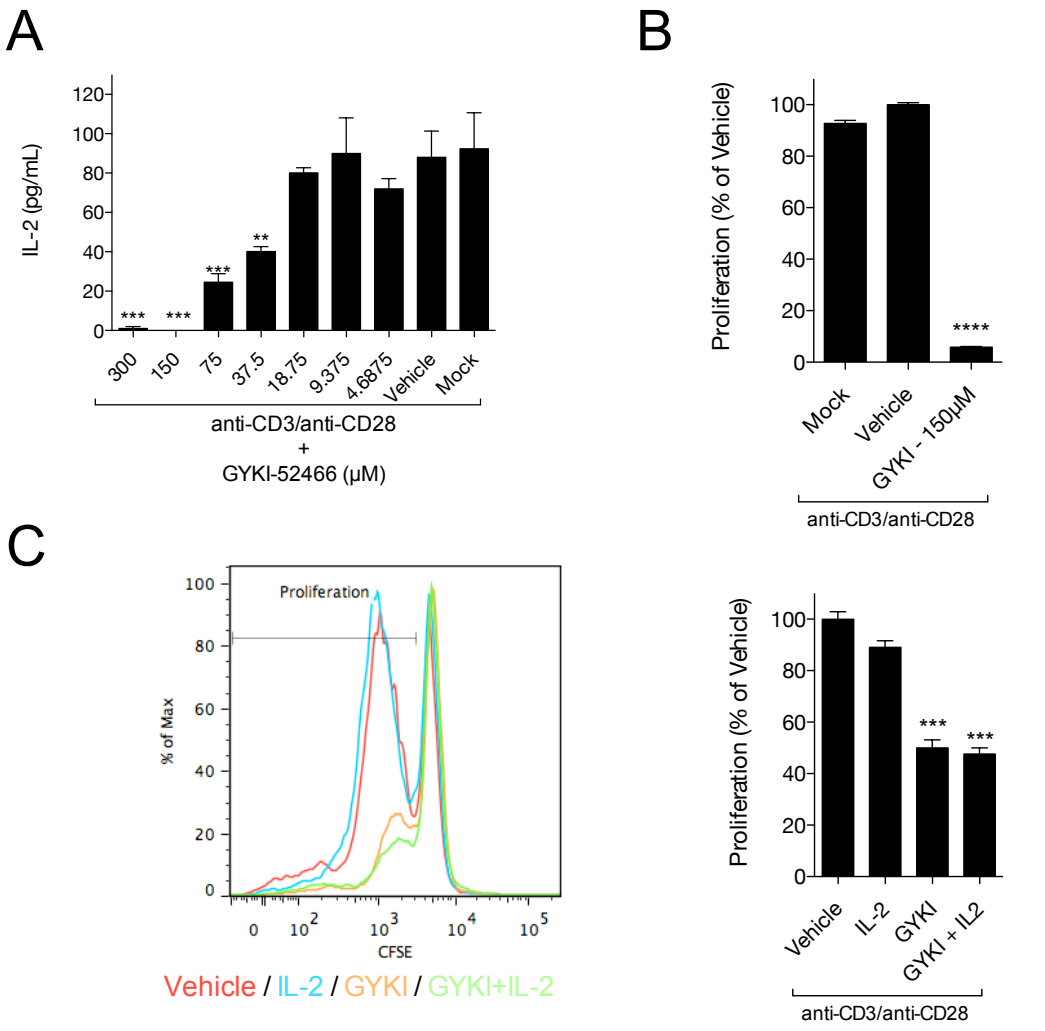


**Figure 4.2:** *GYKI-52466 inhibits IL-2 production in primary lymphocytes* Splenocytes or purified CD3+ cells from CD-1 mice were activated using plate bound anti-CD3/anti-CD28. GYKI-52466 was serially diluted from 300$\mu$M - 0$\mu$M (Vehicle) and IL-2 production measured by ELISA **(A)**. Proliferation was measured by CFSE dilution assay via flow cytometry **(B)**. IL-2 failed to rescue proliferation **(C)** Error bars represent +/- SEM of two replicates per group. **P<0.01,***P<0.001, ****P<0.0001, (One-Way ANOVA).

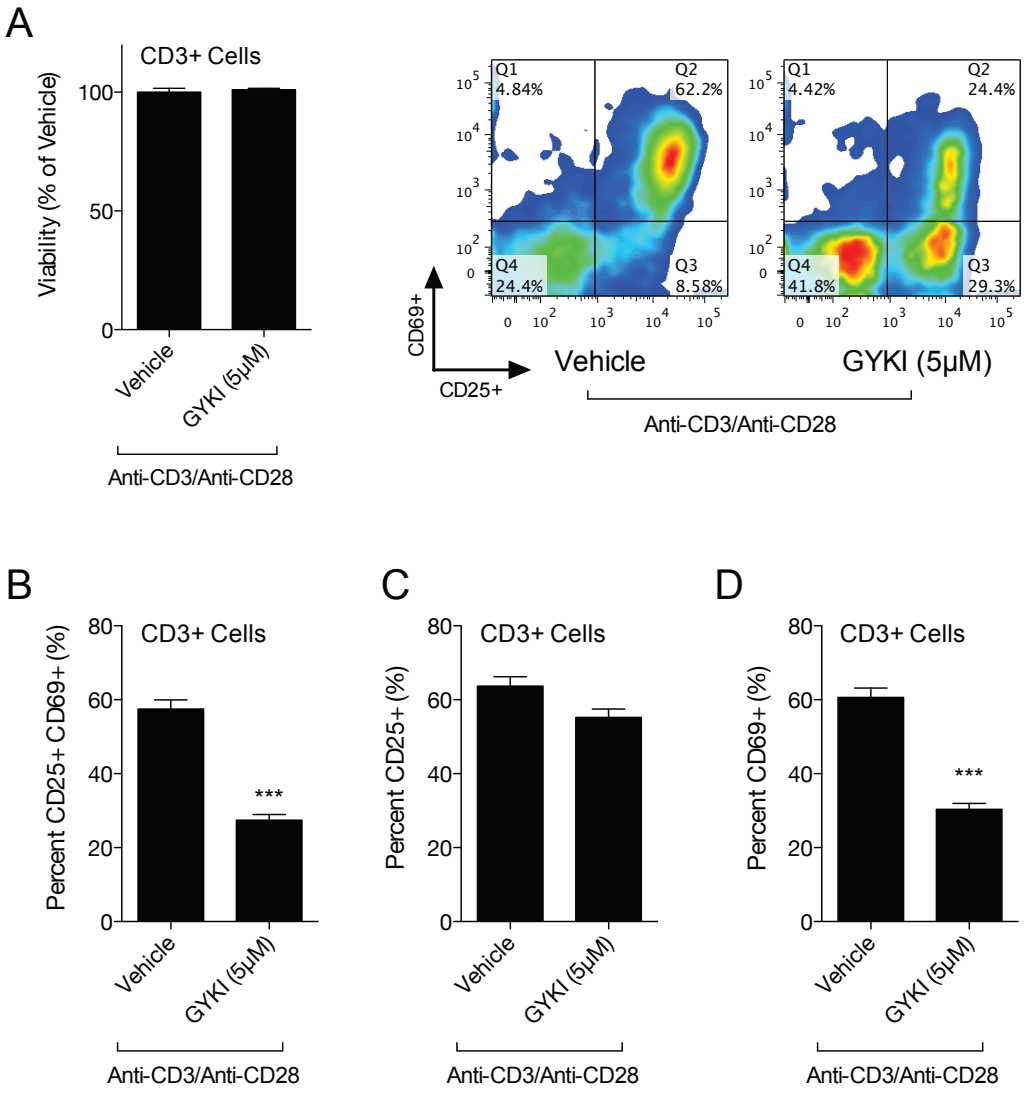


**Figure 4.3:** *GYKI-53655 Treatment inhibits T-cell activation.* Purified CD3+ T-cells were stimulated with anti-CD3/anti-CD28. 12h post stimulation, T-cells were stained for CD69(PE)/CD25(APC) and analyzed by flow cytometry. Percent of double positive **(B)**, CD25+ **(C)**, CD69+ **(D)** were calculated. Error bars represent +/- SEM of mean of three biological replicates. Data are representative of at least two independent experiments. ***P <0.001 (Student's t-test)

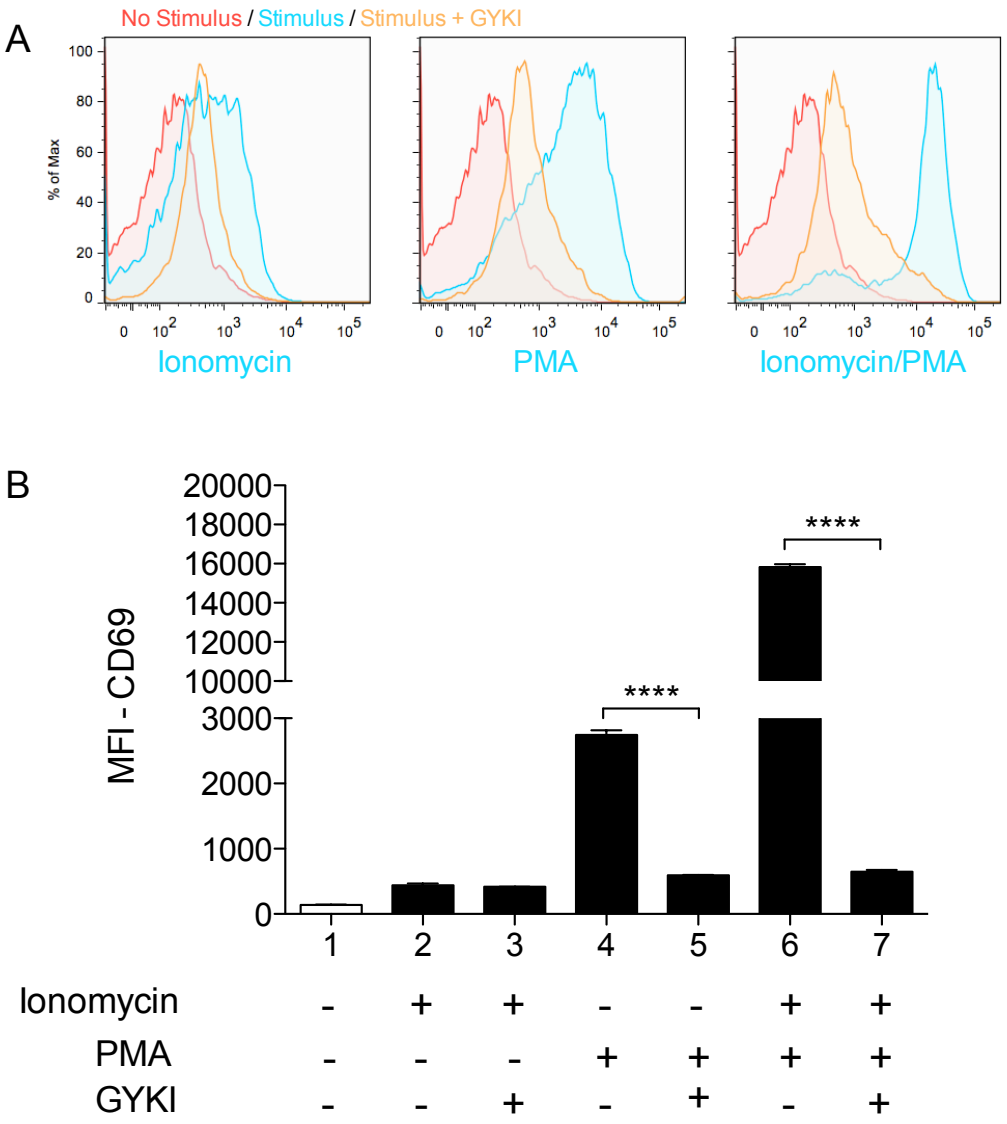


**Figure 4.4:** *GYKI-52466 inhibits PMA induced CD69 expression.* Purified CD3+ CD-1 mice were harvested and stimulated with Ionomycin, PMA, or Ionomycin/PMA. Simulated cells were then treated with GYKI-52466 (150$\mu$M) or vehicle control (0.1M HCl). CD69 expression was measured by flow cytometry. ****P<0.0001, (Student's t-test)

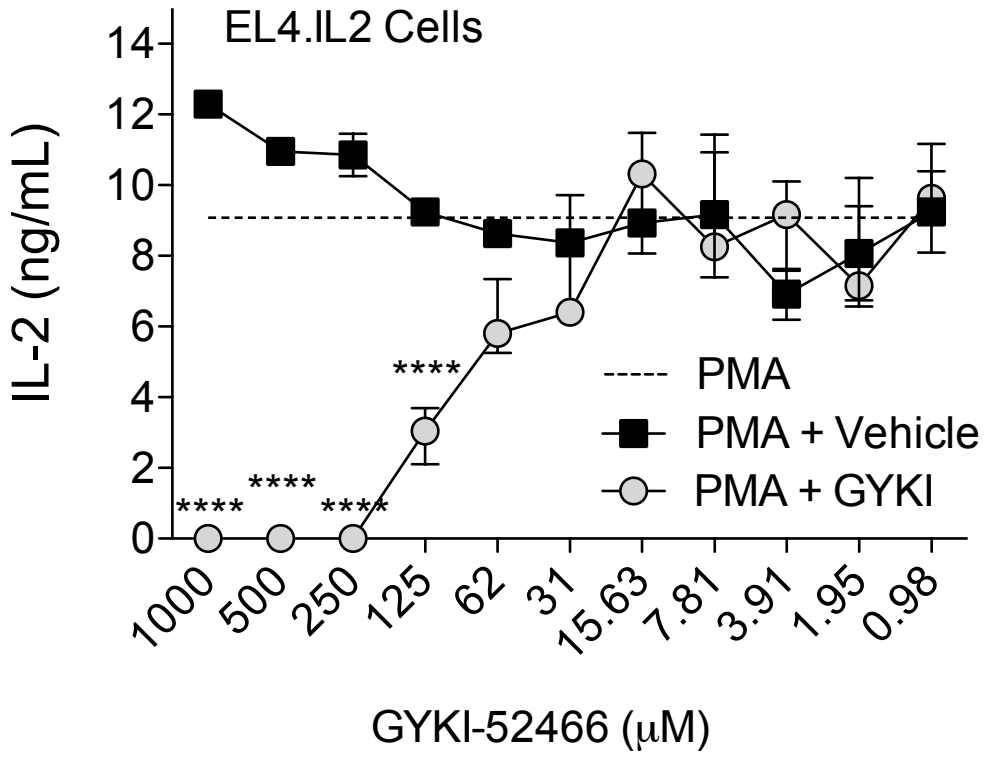


**Figure 4.5:** *EL4.IL2 cells treated with GYKI-52466 have lower IL-2 production* EL4.IL2 cells activated using PMA (20nM). GYKI-52466 was serially diluted from 200$\mu$M - 0$\mu$M. Cells were grown in regular media (DMEM, 10% Regular FBS, Pen/Strep, Glutamine). Cell culture supernatant was collected 12h post activation and analyzed for IL-2 production by ELISA. ****P<0.0001, (One-Way ANOVA).

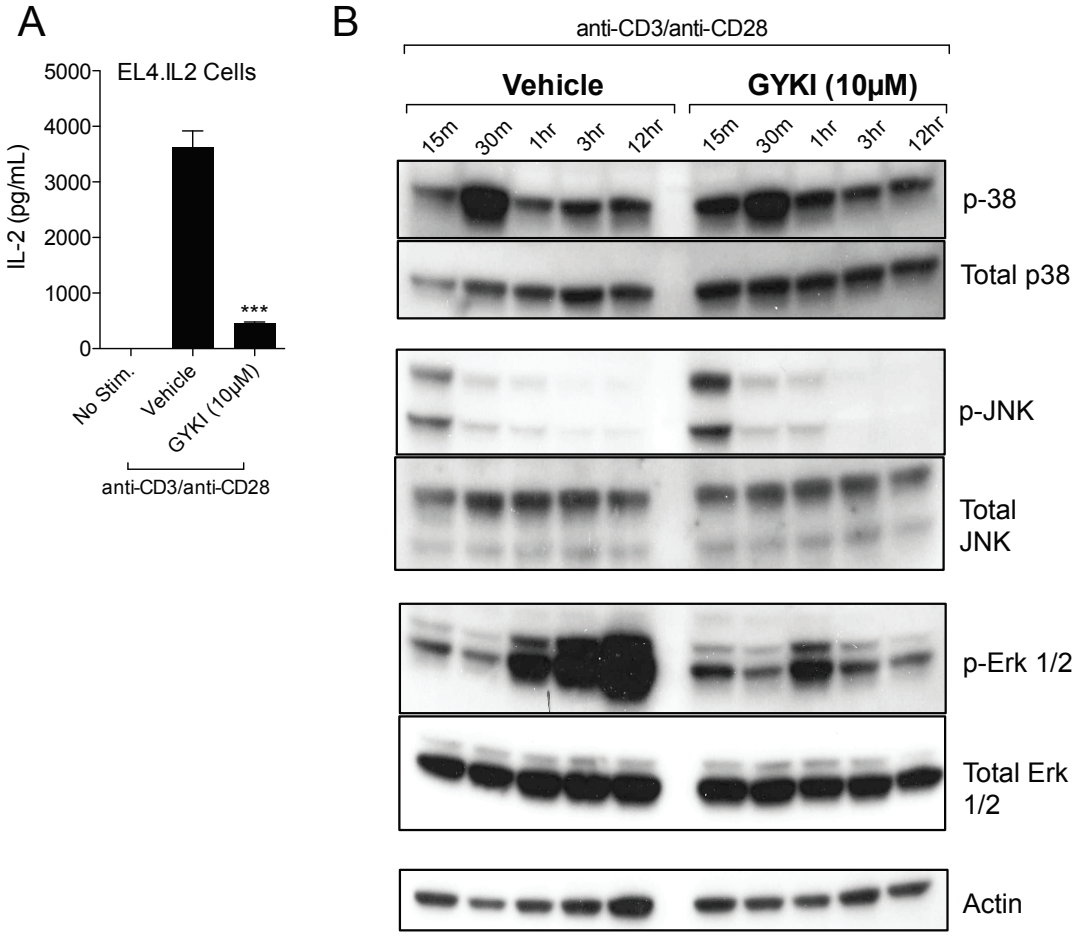


**Figure 4.6:** *GYKI-53655 inhibits sustained Erk activation in EL4.IL2 cell line.* EL4.IL2 cells were stimulated with anti-CD3/anti-CD28. After 12h post stimulation, culture supernatant were analyzed for IL-2 via ELISA **(A)**. GYKI-53655 treated and vehicle treated cells were probed for phospho-p38, phospho-Jnk, phospho-Erk, and actin over a 12 hour time course **(A)**.

with anti-CD3/CD28 antibodies ((Figure 4.1). Purified T-cells treated with GYKI-52466 failed to grow in size or proliferate in vitro. Using a more potent 2, 3 benzodiazepine, GYKI-53655, these effects were evident at low micromolar concentrations (Figure 4.1). The inhibition of CD69 expression (Figure 4.3A, Figure 4.4) suggested that GYKI had an effect on the Ras-Erk pathway [38,165]. Using the EL4.IL2 mouse cell line, we showed that GYKI-53655 inhibited sustained Erk pathway activation and IL-2 production (Figure 4.6).

Interestingly, AMPA receptors are known to be present in various continuously proliferating cell lines [149]. Treating these transformed cells with glutamate receptor antagonists in particular GYKI-52466 inhibited their proliferation [149]. We showed a defect in sustained Erk activation in the EL4.IL2 T-cell line (Figure 4.6) with GYKI treatment. Studies have shown that AMPA receptors activate the MAPK pathway, result in sustained Erk activation [172, 184]. Erk signaling is required for the activation of transcription factor AP-1 involved in IL-2 gene transcription and sustained activation of Erk is necessary for the upregulation of cell cycle cyclins [61, 154, 190]. In one study using a lung cancer cell line, GYKI-52466 treatment inhibited proliferation by inhibiting Erk phosphorylation while treating with an AMPA agonist induced Erk phosphorylation [172, 184].

T-cells express receptors for a variety of neurotransmitters including glutamate receptors [8, 57, 71, 106, 150]. Multiple studies have shown that glutamate receptors of both the ionotrophic and metabotrophic classes can be found in multiple non-CNS tissues such as heart, kidneys, spleen, and pancreas [10, 46, 71, 85]. Peripheral glutamate receptors respond to glutamate and this response is inhibited by their respective antagonists. Others have also found a subset of ionotrophic and metatrophic glutamate receptors including AMPA and NMDA receptor subunits on immune cells

including T-cells, B-cells, thymocytes, and dendritic cells [1, 57, 106, 189]. These receptors control a range of functions including calcium signaling, migration, adhesion, and proliferation. However, these studies have not investigated the effects of allosteric AMPA receptor antagonists on the proliferation of primary T-cells.

Our studies indicate that AMPA receptor antagonists play a novel role in inhibiting T-cell activation and proliferation. Because the IL-2 induction pathway is highly conserved between mice and humans and is a major target of current commercial immunosuppressants such as cyclosporine, this offers an opportunity to investigate potential novel pathway for immunosupression. These studies showed potent immunological effects of a glutamate receptor antagonist that have therapeutic implications for CNS diseases mediated by the immune system and also non-CNS immunological diseases. AMPA receptor antagonists might provide a novel mechanism of immunosuppression with potential benefit in neuroinflammatory disease such as alphaviral induced viral encephalomyelitis where glutamate excitotoxicty and neuroinflammation play a critical role.

# CHAPTER 5

# FINAL DISCUSSION

**Contents**

## 5.1 Final Discussion

Inflammation in the nervous system is a necessary part of the response to CNS infection, but also causes neuronal damage in both infectious and autoimmune diseases of the CNS. We have studied the role of host immune factors in the outcome of viral encephalomyelitis caused by neuronal infection with the mosquito-borne alphavirus, neuroadapted Sindbis virus (NSV) [89,90,147]. Previous studies showed that survival after NSV infection is improved in T-cell deficient mice and in mice with pharmacological inhibition of the inflammatory response indicating that development of treatments that reduce CNS inflammation is an important therapeutic goal [65, 90, 125, 147]. In addition, glutamate excitoxicity is a major mechanism of damage to mature neurons in viral encephalitis. Perturbations in glutamate homeostasis as a result of viral replication [124], neurotoxic cytokines production [21, 141], ineffective clearance [21, 141], production of excess glutamate [26, 77, 191, 195], and changes in glutamate receptor composition [193] can cause excitotoxicity.

Therefore, drugs that protect neurons from glutamate excitotoxicity as well as inhibit the peripheral immune response may provide an option for treatment of viral encephalitis.

### 5.1.1 The immune response and glutamate clearance

Despite being present in neurons at millimolar concentrations intracellularly, levels of glutamate in the extracellular synaptic cleft between neurons are kept low and tightly regulated to control signaling [112]. Astrocytes utilize glutamate transporters, predominately GLT-1 (also known as EAAT2) to remove excess glutamate and keep concentrations at nanomolar levels in the synaptic cleft [162]. Glutamate is then recycled back to glutamine by glutamine synthetase and released into the extracellular space

for uptake by neurons (Figure 1.3B) [20, 130, 131]. The failure of glutamate transporters to clear glutamate and keep glutamate concentrations low in the interstitial space between neurons can lead to neurodegeneration [146]. Acute inflammation, as occurs in viral encephalitis, can cause perturbations in glutamate clearance mediated by inflammatory cytokines [170].

NSV infection induces downregulation of GLT-1 on astrocytes indirectly through the actions of inflammatory cytokines, IL-1$\beta$ and TNF-$\alpha$ [21, 140]. BALB/c mice resistant to NSV-induced fatal disease have lower levels of IL-1$\beta$ and TNF-$\alpha$ and better glutamate clearance during NSV infection. Mice deficient in IL-1$\beta$ and TNF-$\alpha$ were protected against NSV-induced mortality and paralysis. TNF-$\alpha$ deficient mice did not show the defects in glutamate transport during NSV infection seen in wild type mice [21] due to preservation of homeostatic levels of the glutamate transporter GLT-1 [21]. Similarly, NSV-infected, IL-1$\beta$-deficient mice showed limited spinal cord motor neuron degeneration and limited decrease of the GLT-1 transporter in the spinal cord [141]. We showed that DON-treated mice protected from paralysis and death had significantly lower levels of IL-1$\beta$ and TNF-$\alpha$ mRNA in the brain (Figure 2.8). Once DON treatment was stopped, IL-1$\beta$ and TNF-$\alpha$ mRNA expression increased paralleling the increase in paralysis and mortality (Figure 2.8). DON treatment might contribute to preserving GLT-1 during NSV infection, thus enabling glutamate clearance.

Immunodeficient SCID mice have lower levels of IL-1$\beta$ and TNF-$\alpha$ during NSV infection compared to immunocompetent mice [188]. This suggests that the adaptive immune response might play a role in aggravating IL-1$\beta$ and TNF-$\alpha$ levels by microglia or macrophages during NSV infection. This hypothesis is supported by comparison of innate cytokine mRNA expression in the brains of DON-treated and

PBS-treated mice. Peak IL-1$\beta$ production occurred on day 5 which coincided with increased infiltration of T-cells into the CNS and peak IFN-$\gamma$ production. During DON treatment both T-cell infiltration and IL-1$\beta$ production were ablated. (Figure 2.8 ) After halting DON treatment, IL-1$\beta$ mRNA started to increase which was coincident with CD3+ lymphocyte infiltration into the brain (Figure 2.7, Figure 2.8 ). A recent study with EAE (experimental autoimmune encephalomyelitis), a model for multiple sclerosis, showed that brain-infiltrating CD3+ lymphocytes, in addition to activated macrophages, were a potent source of IL-1$\beta$. Purified antigen-specific T-cells in EAE produced 10-fold higher IL-1$\beta$ in culture. of these lymphocytes with brain slices showed increased excitatory postsynaptic currents which were ablated by IL-1 receptor antibody treatment [111]. In mice, treatment with IL-1 receptor antibody limited EAE clinical disease and delayed mortality [111]. Similarly, in NSV infection continuous exogenous treatment with IL-1$\beta$ receptor antibody reduced paralysis and delayed death [79] suggesting that inhibiting IL-1$\beta$ alone might not be sufficient to prevent mortality in NSV-induced disease.

NSV-infected mice treated continuously with the neuroprotective drug, minocycline, inhibited the production IL-1$\beta$ and TNF-$\alpha$ by microglia and were rescued from fatal paralysis, but delayed treatment reduced the efficacy of this intervention [79]. However, in addition to reducing microglia activation, minocycline has a variety of effects including inhibition of T-cell proliferation [87, 92, 110]. NSV-infected mice treated with minocycline had less microglia cell activation and decreased CD45+ cell infiltration [79]. NSV clearance from the brain and spinal cord was not affected suggesting that antibody responses might be intact [79]. Limited lymphocyte infiltration might have contributed to protection by limiting increases of neurotoxic cytokines IL-1$\beta$ and TNF-$\alpha$.

How the IL-1$\beta$-producing inflammasome is triggered in neurons and microglia by neurotrophic viruses is still an area of intense study [24]. Though inhibiting IL-1$\beta$ production might be beneficial in NSV infection, studies of the mouse model of West Nile virus (WNV) infection show that inhibiting IL-1$\beta$ production exacerbates pathology resulting in reduced survival, increased viral burden, and an exacerbated inflammatory response in the brain [96]. However, disruption of glutamate clearance mechanisms has also been implicated in WNV flaccid paralysis [7] and neurotrophic human coronavirus infection in mice (HCoV) [13]. Similar to NSV, glial activation, IL-1$\beta$ and TNF-$\alpha$ production have been associated with neuronal cell death in Japanese encephalitis virus (JEV) infection [27, 28, 41, 160]. In the model for lethal herpes simplex virus type 1 (HSV-1) encephalitis both IL-1$\beta$ and TNF$\alpha$ have a protective phenotype, limiting viral load, viral dissemination, and facilitating the induction of the immune response [153]. Patients treated with commercially avaiable anti-TNF-$\alpha$ antibody, Adalimumab, for rheumatologic disorders show increased risk for HSV-1 and varicella zoster virus-induced encephalitis [11, 14]. Moreover, the inability to efficiently clear glutamate has been in implicated in a wide range of non-viral diseases including ALS, Alzheimer's, Parkinsons, Huntington's, ischemia, and traumatic brain injury [192].

These studies suggest that levels of IL-1$\beta$ and TNF-$\alpha$ play an important role in modulating glutamate clearance during NSV viral encephalitis. Inhibiting IL-1$\beta$ and TNF-$\alpha$ might be beneficial in reducing neuronal cell death in alphavirus induced encephomyelitis.

### 5.1.2 The immune response and glutamate generation

Glutamine metabolism is important in neuroinflammatory diseases. Glutamine is utilized by the CNS to synthesize the neurotransmitter glutamate and in the immune system as an essential metabolite for growth and proliferation of lymphocytes in response to antigenic stimulation [109, 126]. In infection with NSV, glutamate excitotoxicity and the adaptive immune response are implicated in neuronal damage.

Viral infections can increase glutamine metabolism and glutamate production by microglia, the resident macrophages of the CNS [26, 77]. Evidence from other CNS virus infections has shown that the conversion of glutamine to glutamate is markedly increased in neuronal and microglial cells during a variety of neuroinflammatory diseases including viral encephalitis [26, 77]. In microglia, astrocytes, and macrophages type I interferons, in particular IFN-$\alpha$, induce transcriptional upregulation of a splice variant of GLS1 (glutaminase), GAC, by phosphorylation of the STAT1 transcription factor, transduction, and subsequent binding to the GLS1 promoter. GLS1 promoter regions of both rodent and human genes have putative STAT1 binding sites. Thus, IFN-$\alpha$ in the CNS can increase GLS1 synthesis and glutamate production.

Additionally, IL-1$\beta$ and TNF-$\alpha$ upregulate GLS1 expression in neurons and microglia resulting in the excess production of glutamate causing autocrine and paracrine glutamate excitotoxicity [26, 133, 191]. DON treatment of microglia infected with Japanese encephalitis virus limited TNF-$\alpha$-induced neurotoxic glutamate production. In this dissertation, we showed that treatment with DON limited the production of IL-1$\beta$ and TNF-$\alpha$ in the brains of NSV-infected mice (Figure 2.8) possibly indirectly limiting the production of excess glutamate.

Infiltrating immune cells can also exacerbate glutamate excitotoxicity in the CNS

by providing an additional source of non-neuronal glutamate. In particular, activated infiltrating CD8+ T-cells are a potent source of non-neuronal glutamate [114]. Inhibiting glutamine metabolism may also prevent the generation of glutamate from these non-neuronal cells [26, 77, 195]. Because glutamine metabolism is also involved in lymphocyte proliferation, glutamine antagonists might prove beneficial in limiting CNS immune cell infiltration as well as directly curbing the production of glutamate by lymphocytes already in the CNS.

GLS1 is up regulated during lymphocyte activation and is a major player in glutamine metabolism and signal transduction. Silencing GLS1 via siRNA or pharmacologically inhibiting GLS1 [33] through use of GLS1-specific small molecules (e.g. JHU-212, Appendix Figure B.3) inhibits lymphocyte proliferation. Moreover, small molecule inhibition of GLS1 in microglia inhibits neurotoxic glutamate production. Because GLS1 plays a critical role in the nervous systems as well as in the peripheral immune system, development of GLS1-specific inhibitors might offer a more specific and targeted way to limit the generation of neurotoxic glutamate production in the CNS as well as limit peripheral lymphocyte proliferation in neuroinflammatory disease. However, development of GLS1 specific inhibitors that are effective in vivo have been plagued by solubility, stability, and specificity issues. BPTES is a very well characterized GLS1 inhibitor but is highly insoluble [42, 169]. Developing a soluble, BBB permeable, and stable GLS1 inhibitor might be a way to prevent immune-induced neuroinflammatory disease when glutamate excitotoxicity is involved [70].

### Improving DON

We observed that continuous treatment with DON even at low doses to be toxic. Because DON is a competitive glutamine inhibitor, one approach to limit the toxicity

of DON would be to reduce levels of extracellular glutamine. A recent Phase IIb study using DON in conjunction with PEGylated glutaminase to deplete serum glutamine levels showed an increase in effectiveness and tolerability of DON for treatment of late stage colorectal and lung cancer [86, 122, 180]. Because lymphocytes are sensitive to extracellular glutamine concentrations [23], depletion of plasma glutamine using this combinatorial approach might be beneficial in the treatment of neuroinflammatory and other inflammatory diseases by reducing the dose of DON required to inhibit lymphocyte proliferation in vivo.

Analogously, we hypothesize that the pharmacological depletion of serum glutamine using high doses of sodium phenylbutyrate, a drug used for treatment of urea cycle disorders, in conjunction with DON might have a similar effect on increasing DON efficacy and tolerability [40]. Because glutamine in the CNS is derived from glucose, serum glutamine depletion should not affect intrinsic CNS glutamate homeostasis. Additionally, serum glutamine depletion alone can be beneficial in limiting the proliferation of lymphocytes, because proliferating lymphocytes are highly sensitive to extracellular glutamine concentrations [23]. This approach can decrease inflammation by inhibiting induction of the peripheral adaptive immune response.

However, the glutamine serum depletion strategy does not affect generation of neurotoxic glutamate by microglia. DON has limited blood brain permeability thus, its actions might be limited to inhibiting the peripheral immune response and might not directly inhibit the generation of glutamate in the CNS [138]. Modifying DON to make it more BBB permeable or incorporating DON with blood brain drug carriers might beneficial in getting DON into the CNS. We showed that a structurally distinct glutamine antagonist, acivicin ($\alpha$-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), was effective in reducing paralysis and morality in NSV-infected mice (Appendix

Figure 2.2 ). Acivicin has low lipophilicity but can readily pass through BBB by the use of large amino acid transporters [2, 29]. Acivicin might be a good alternative to DON where direct inhibition of glutamate production by neuronal and microglial GLS1 is necessary.

### 5.1.3 Protecting Neurons - Inhibiting response to glutamate

Excess glutamate generation or reduced clearance can lead to glutamate excitotoxicity by promiscuously activating glutamate receptors on neurons that induce signaling cascades that result in neuronal death by apoptosis or necrosis. Necrotic death results in further release of intracellular neuronal glutamate and can contribute to bystander neuronal death of uninfected nearby neurons during SINV infection [39, 124]. Preventing glutamate signaling by allosterically inhibiting AMPA receptors is beneficial in protecting cortical, hippocampal, and especially spinal cord motor neurons in NSV-induced disease [125]. Motor neurons have calcium permeable AMPA receptors making them sensitive to glutamate excitotoxicity. NSV infected neurons treated with NASPM, a selective antagonist of calcium permeable AMPA receptors, altered pathogenesis in rats [39].

GYKI-52466 is the prototypic AMPA receptor antagonist of the 2,3 benzodiazepine class that allosterically inhibits AMPA receptors [161]. Inflammatory cytokines such as IL-1$\beta$ and TNF-$\alpha$ can cause the redistribution of calcium permeable AMPA receptors during neuroinflammatory disease, increasing sensitivity of cells to glutamate excitotoxicity [193]. Mice treated with AMPA receptor antagonists, GYKI-52466 or Talampanel, but not NMDA receptor antagonists survived infection with limited paralysis [65, 125]. Similar to DON treatment, AMPA receptor antagonists limited peripheral lymphocyte proliferation, subsequent lymphocyte infiltration into

the CNS, and as a result delayed viral clearance. This further implicates the adaptive immune response in morbidity and mortality during NSV infection.

In this dissertation we showed that AMPA receptor antagonists have a direct effect on the activation of the peripheral immune response. We showed that these neurotrophic drugs had a direct effect on primary lymphocytes. CD3+ lymphocytes treated with GYKI-53655, a potent analog of GYKI-52466, showed inhibition of T-cell activation *in vitro*. GYKI-53655 inhibited the production of T-cell growth factor, IL-2, and inhibited the sustained activation of Erk, a critical downstream MAPK necessary for T-cell activation and proliferation [60].

In infection with neurotrophic human coronavirus (HCoV, causative agent of SARS) infection in mice, treatment with GYKI-52466 limited microglia but not astrocyte activation. Treatment limited neuronal damage, rescued GLT-1 expression on astrocytes, and rescued mice from paralysis but not mortality. However, unlike in NSV infection, GYKI-52466 treatment did not have any effect on the viral replication [12]. In this study, GYKI-52466 was used at 3mg/kg twice daily while in our previous studies with NSV, GYKI-52466 was used at 10 mg/kg twice daily [125]. In vitro studies with primary lymphocytes, showed that high doses of GYKI-52466 can inhibit lymphocyte activation and proliferation (Appendix Figures C.1-4.5. Using the high affinity derivative, GYKI-53655 (Chapter 4), we replicated primary lymphocyte inhibition at significantly lower concentrations (Figure 4.1-4.3). Studies show protective and pathological effects of the immune response in the HCoV model [**?**, 9, 18]. Immunodeficient mice have delayed mortality, while mice treated with immunsupressive drug, cyclosporine, show increased mortality [18, 83]. It would be interesting to see if using a higher dose of GYKI-52466 would rescue these mice from death higher doses protect neurons and inhibit peripheral inflammation in the NSV model [125].

In total, the studies suggest that AMPA induced glutamate excitotoxicty plays a role during alphaviral encephalomyelitis and that AMPA receptors antagonists protect neurons by blocking glutamate excitotoxicty as well as neuroinflammation.

### 5.1.4 Summary

In this dissertation we showed that DON protects mice from NSV-induced fatal encephalomyelitis by inhibiting the induction of the peripheral immune response and preventing CNS inflammation. DON and similar glutamine antagonists could have an ancillary role in preventing glutamate excitotoxicity by inhibiting the generation of glutamate from neuronal and non-neuronal cells. This might make DON an ideal candidate drug for treating diseases where glutamate excitotoxicity and inflammation play a role. In addition we revealed a novel effect of AMPA receptor antagonists on lymphocyte proliferation by inhibiting T-cell activation through the Erk signaling pathway resulting in limited IL-2 production.

In total, these studies show that treatment of alphavirus induced encephalomyelitis requires multiple approaches. Glutamate toxicity, immune cell infiltration, and viral clearance must be addressed to prevent damage and allow clearance of virus from the CNS. Currently, drugs like DON and AMPA receptor antagonists prevent neuronal damage but do not help clear virus. Immunomodulatory approaches such as selectively biasing the qualitative nature of the immune response to mitigate damage and facilitate clearance must be investigated to provide complete clearance and control.

# APPENDIX A

# APPENDIX - CHAPTER 2

**Contents**



**Figures**



## A.1 Supplementary Methods

### A.1.1 Western Blot Analysis

Brain homogenates were prepared in PBS from vehicle and drug-treated NSV-infected mice as described above. Homogenates were diluted in 2X RIPA (Cell Signaling) buffer containing protease and phosphatase inhibitors (Sigma), incubated at 4℃ for 15 minutes and then clarified by centrifugation for 30min at maximum speed in a

microcentrifuge in the cold. Protein concentration was determined using the DC Assay (BioRad) and samples were boiled in Laemli buffer. Samples were either stored at -80℃ or were immediately loaded (20-40$\mu$g) onto 4-20% gradient gels for electrophoresis. Samples were then transferred onto nitrocellulose membranes (BioRad) and stained using Ponceau S (Sigma) to determine efficient transfer and loading. The membrane was blocked for 1h at room temperature in 5% non-fat dry milk (5% milk in TBS + 0.1% Tween-20), washed for 5min in TBST (TBS with 0.1% Tween-20), and incubated at 4℃ overnight with rabbit antibody against GFAP (1:1000, Cell Signaling) or caspase-3 (1:1000, Cell Signaling) in TBST with 5% BSA. After washing three times for 10 minutes in TBST, the membrane was incubated with anti-rabbit IgG (1:4000, GE Amersham) for one hour at room temperature in TBST with 1% nonfat dry milk and washed five times with TBST for 10 minutes. The membrane was then incubated in ECL reagent (GE Amersham) and developed on film (GE Amersham).

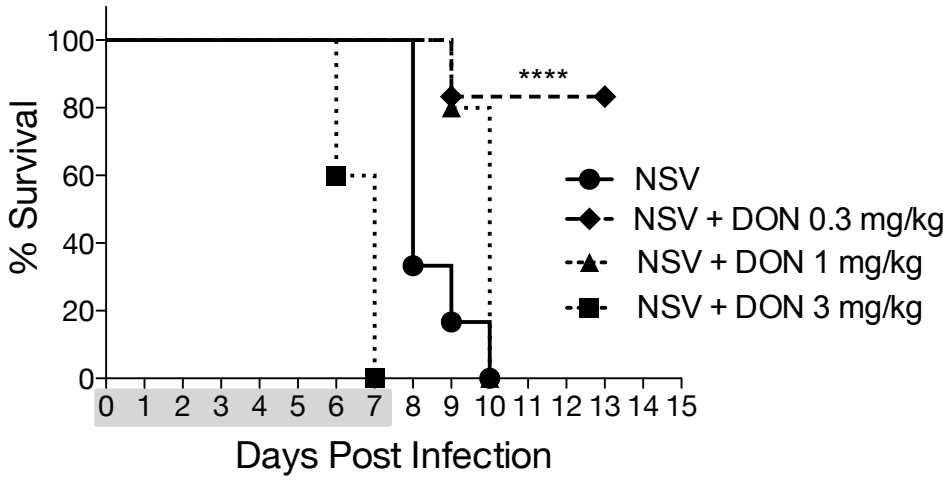


**Figure A.1:** *High dose DON is toxic to mice.*
C57BL/6 mice were infected with 1000 pfu of NSV intracerebrally and treated every 24h intraperitoneally with glutamine antagonist DON (3 mg/kg, 1 mg/kg, 0.3 mg/kg) or PBS vehicle (100 - 200$\mu$L) i.p. through day 7. For untreated mice (NSV) median survival was 8 days. Mice treated with DON at 3 mg/kg and 1 mg/kg had a median survival of 7 and 10 days, respectively. Mice treated with DON at 0.3 mg/kg had 80% survival. ****P <0.0001; Log-rank (Mantel-Cox) survival test.

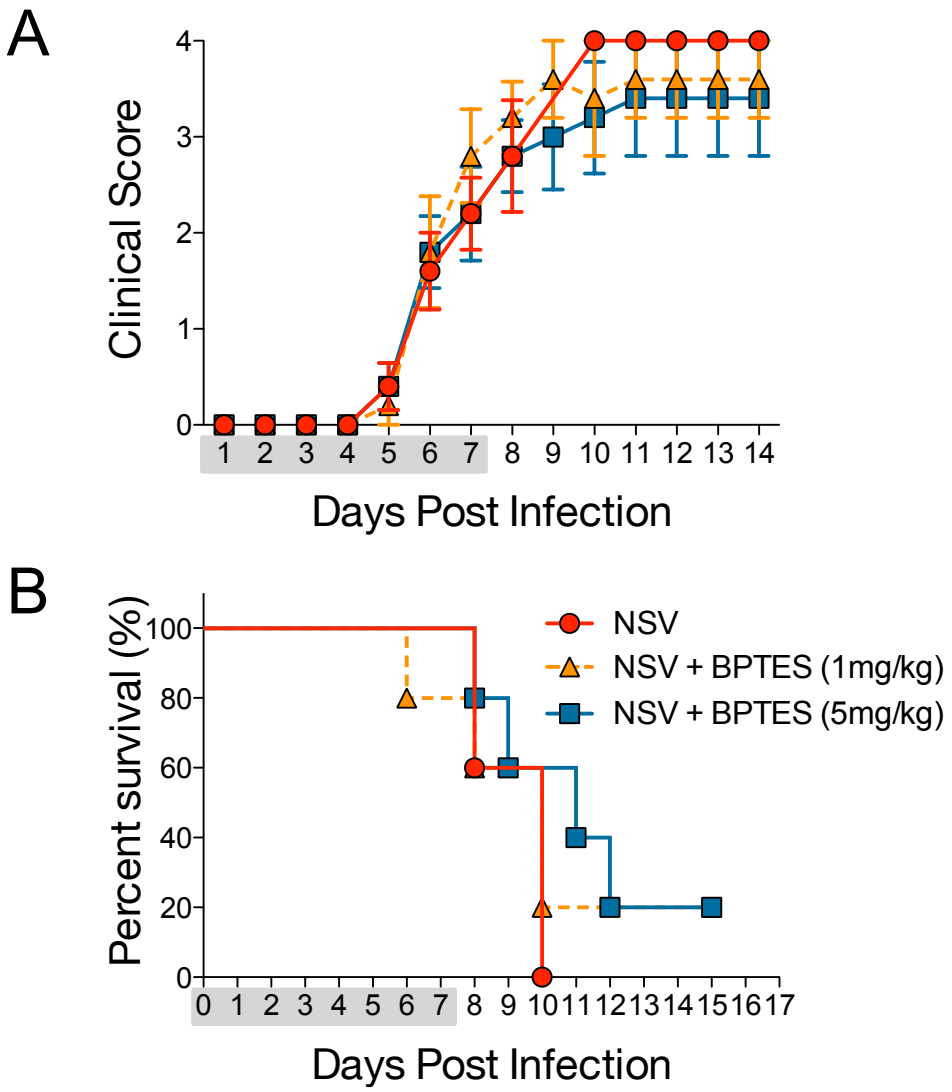


**Figure A.2:** *Effect of glutaminase (GLS1) antagonist, BPTES, on NSV infected mice*

Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) is a GLS1 antagonist that allosertically inhibits kidney-type glutaminase isoform, GLS1. C57BL/6 mice (N=5 per group) were infected with 1000 pfu of NSV intracerebrally and treated every 24h intraperitoneally with BPTES ( 5mg/kg, 1 mg/kg) or Vehicle (20% $\beta$-cyclodextrin in PBS) i.p. through day 7. There were no differences in mortality or clinical score in treated and untreated mice. Log-rank (Mantel-Cox) survival test.

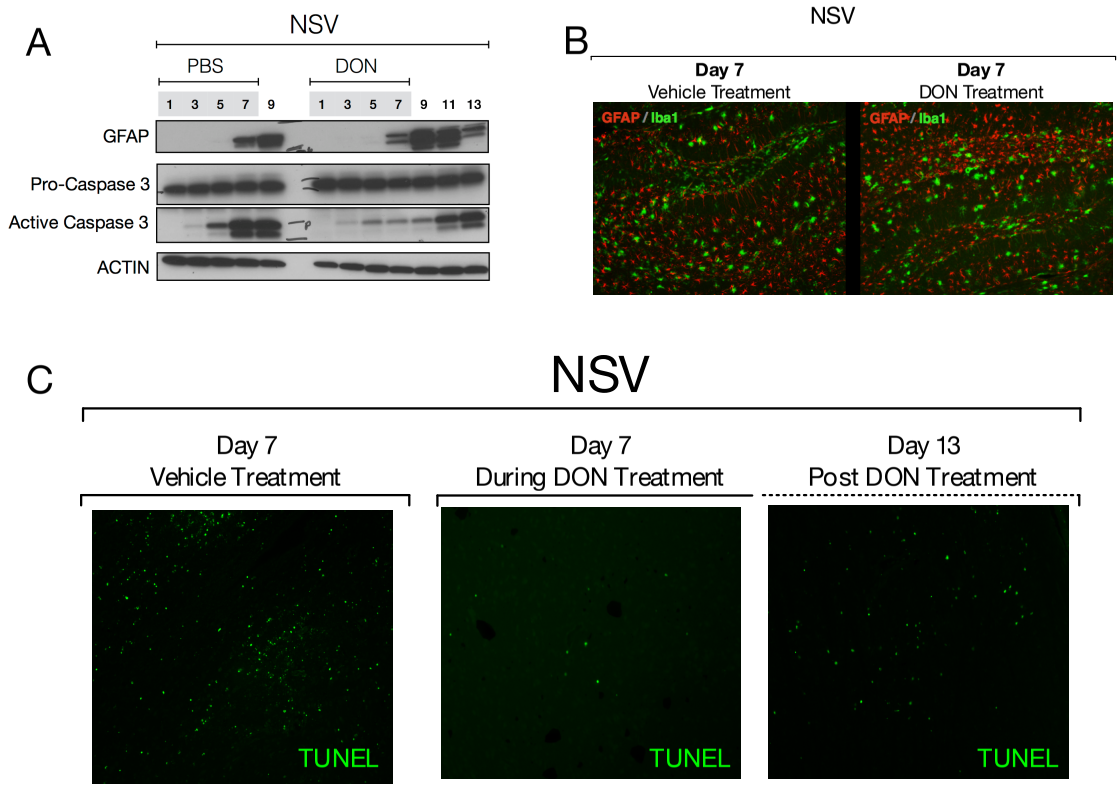


**Figure A.3:** *Effect of DON treatment on GFAP activation and cell death in the CNS*
Western blot of brain homogenates of DON treated and untreated mice (PBS) for GFAP, Caspase-3, and Actin **(A)**. There were no differnece in Iba1 and GFAP levels in DON treated and Vehicle treated mice on Day 7 **(B)**. TUNEL staining of brain sections of mice treated with DON (0.3 mg/kg) or Vehicle (PBS) during and post-treatment (Day 13) **(C)**.

# APPENDIX B

# APPENDIX - CHAPTER 3

## Contents



## Figures

## B.1 Supplementary Methods

### B.1.1 Drugs

DON (Sigma) was dissolved in PBS to produce a 100mM stock concentration and stored in -80℃. Glutaminase (GLS1) inhibitor JHU212 (BSi) and its inactive analog JHU-365(BSi) were dissolved in DMSO to produce a 100mM stock and stored at -20℃.

### B.1.2 GLS1(+/-) Mice

GLS1(+/-) mice were obtained from the BSi (Brain Science Institute, Johns Hopkins School of Medicine). CD3+ lymphocytes were isolated and stimulated as previously described (See 3.2.2). CD3+ T-cells were treated with a suboptimal dose of JHU-212 (2.5$\mu$M).

### B.1.3 Western Blot Analysis

Homogenates of CD3+ lymphocytes were prepared in RIPA buffer. Homogenates were diluted in 2X RIPA (Cell Signaling) buffer containing protease and phosphatase inhibitors (Sigma), incubated at 4℃ for 15 minutes and then clarified by centrifugation for 30min at maximum speed in a microcentrifuge in the cold. Protein concentration was determined using the DC Assay (BioRad) and samples were boiled in Laemli buffer. Samples were either stored at -80℃or loaded (5-15$\mu$g) onto 4-20% gradient gels for electrophoresis. Proteins were then transferred onto nitrocellulose membranes (BioRad) and stained using Ponceau S (Sigma) to determine efficient transfer and loading. The membrane was blocked for 1 hour at room temperature in 5% BSA (5% BSA TBS + 0.1% Tween-20), washed for 5 minutes in TBST (TBS with 0.1% Tween-20), and incubated at 4℃ overnight with Cell Signaling's Pathscan Antibody Cocktail

(1:1000, Cell Signaling) or caspase-3 (1:1000, Cell Signaling) in TBST with 5% BSA. After washing three times for 10 minutes in TBST, the membrane was incubated with anti-rabbit IgG (1:4000, GE Amersham) for one hour at room temperature in TBST with 1% BSA and washed five times with TBST for 10 minutes. The membrane was then incubated in ECL reagent (GE Amersham) and developed on film (GE Amersham).

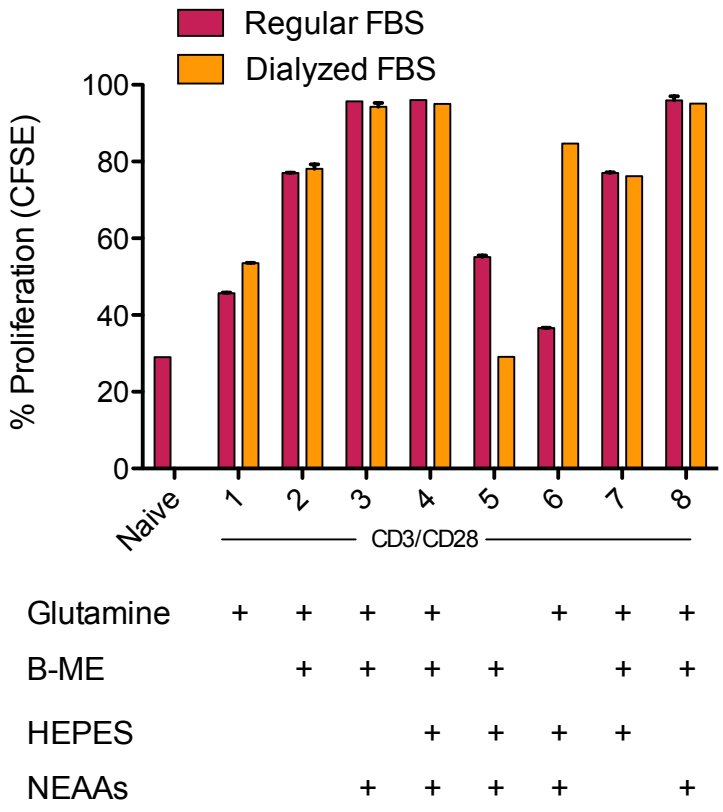


**Figure B.1:** *Glutamine deprivation inhibits lymphocyte proliferation.* CD3+ lymphocytes were isolated from the spleens of C57BL/6 mice and activated using platebound anti-CD3/anti-CD28 (Stim.) and different media formulations. Proliferation was assessed by CFSE dilution 72h post activation. Error bars represent +/- SEM of two replicates per group.

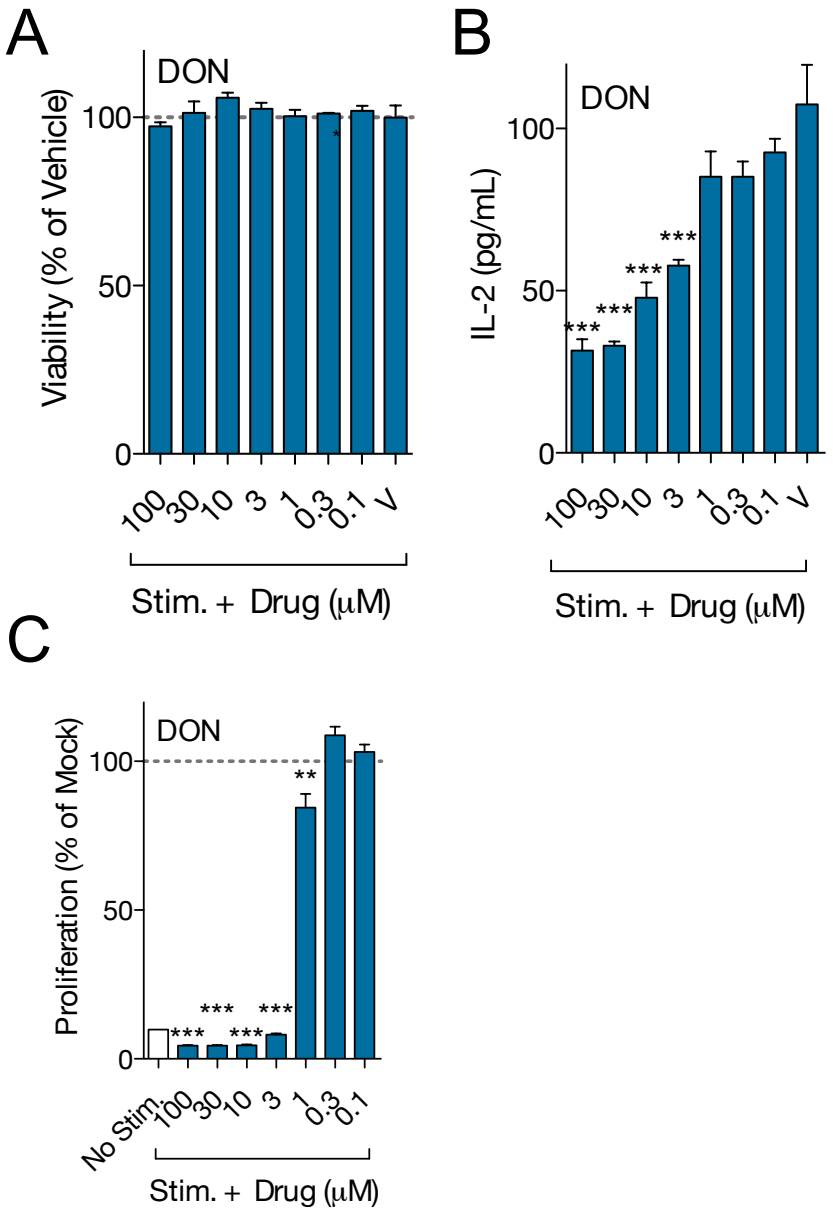


**Figure B.2:** *Effect of different doses of DON on lymphocyte viability, IL-2 production, and proliferation.*
CD3+ lymphocytes were isolated from the spleens of C57BL/6 mice and treated with glutamine antagonist, DON at half-log dose intervals. CD3+ cells were activated using platebound anti-CD3/anti-CD28 (Stim.). Viability was assessed 12-hours post activation **(A)**. Supernatant fluids were collected 12-24h post activation and analyzed for IL-2 production by ELISA **(B)**. Proliferation was assessed by CFSE dilution 72h post activation relative to vehicle or mock controls **(C)**. Error bars represent +/- SEM of three replicates per group. **P<0.01; ***P<0.001, (One-Way ANOVA).

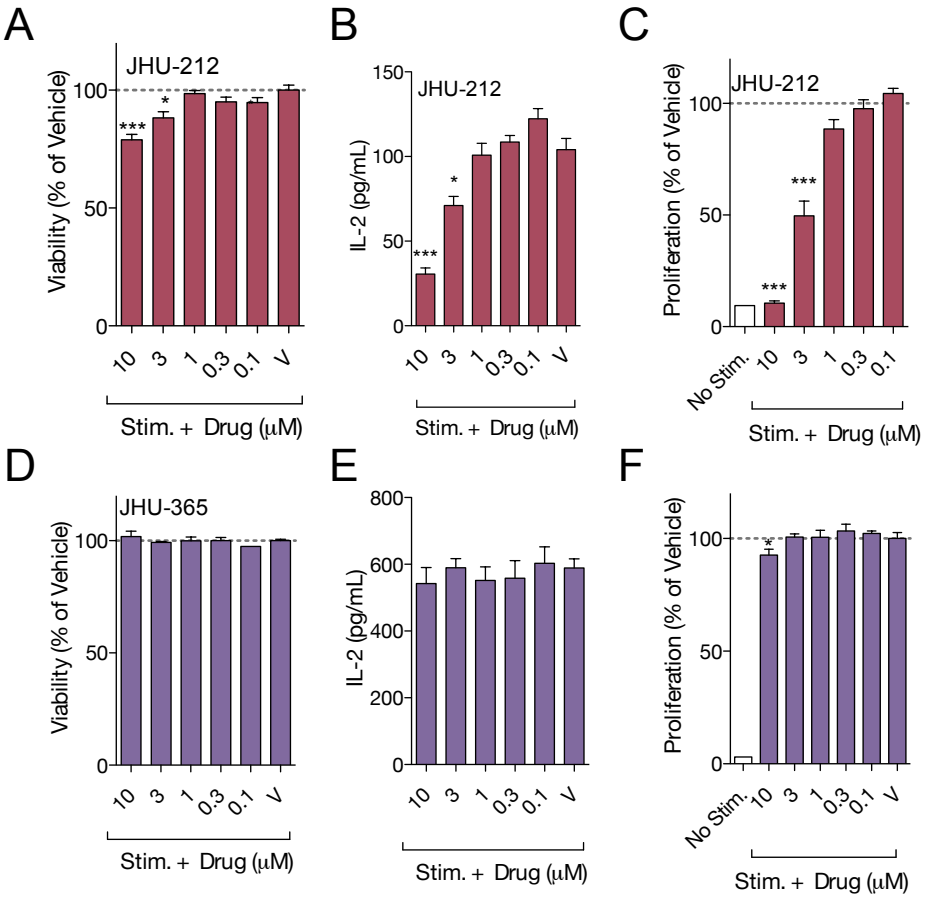


**Figure B.3:** *Effect of different doses of JHU-212 and JHU-365 on lymphocyte viability, IL-2 production, and proliferation*
CD3+ lymphocytes were isolated from the spleens of C57BL/6 mice and treated with glutamine glutaminase (GLS1) inhibitor, JHU212 or its inactive analog JHU-365 at half-log dose intervals. CD3+ cells were activated using platebound anti-CD3/anti-CD28 (Stim.). Viability was assessed 12-hours post activation **(A,D)**. Supernatant fluids were collected 12-24h post activation and analyzed for IL-2 production via ELISA **(B,E)**. Proliferation was assessed by CFSE dilution 72h post activation relative to vehicle or mock controls **(C,F)**. Error bars represent +/- SEM of three replicates per group. *P<0.05; ***P<0.001, (One-Way ANOVA).

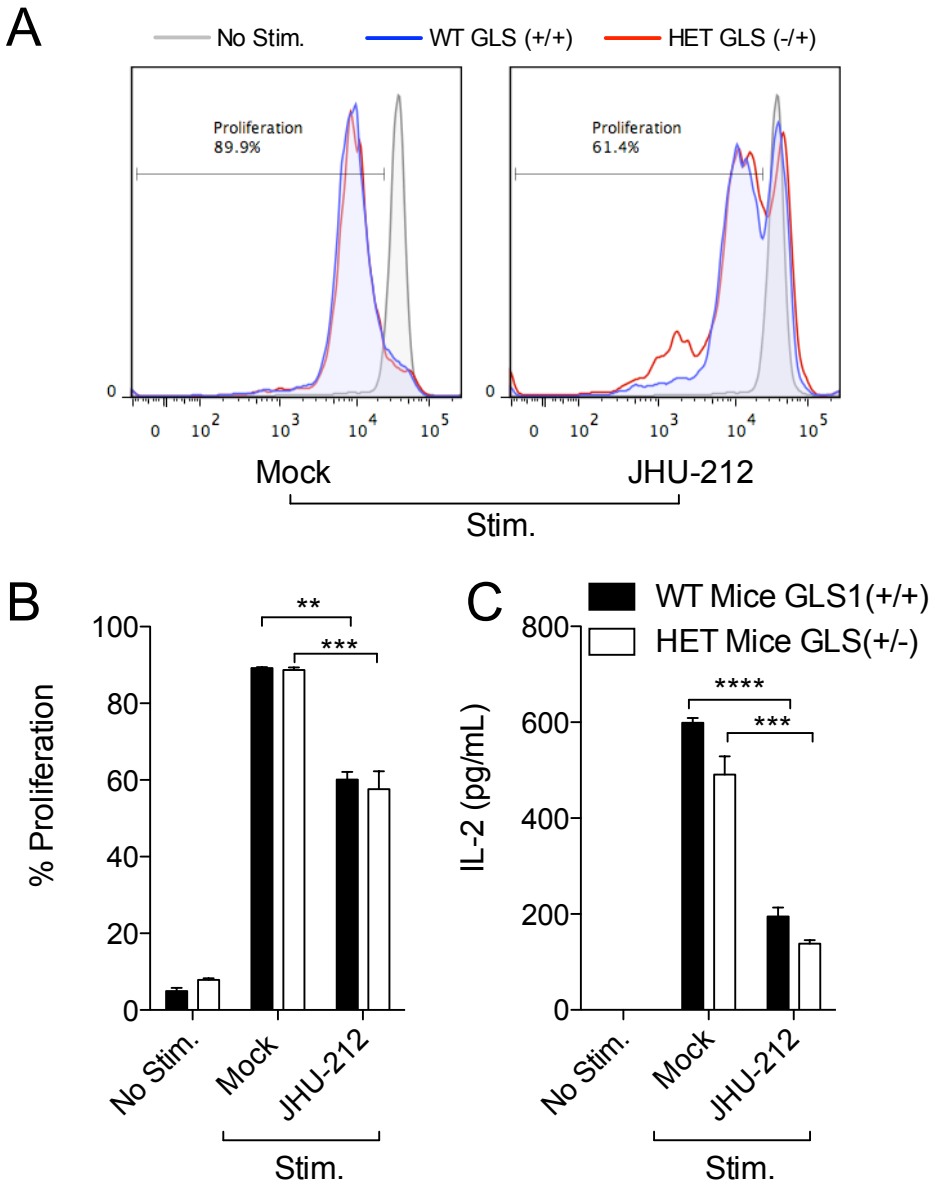


**Figure B.4:** *Effect of hetrogenous GLS1 gene expression on lymphocytes proliferation and IL-2 production.*
GLS(-/+) have reduced GLS1 activity [113]. CD3+ lymphocytes were isolated from the spleens of GLS1(+/+) and GLS1(-/+) mice and stimulated (Stim.) with anti-CD3/anti-CD28. Proliferation was assessed by CFSE dilution 72h post activation **(A-B)** and supernatant fluids for IL-2 ELISA were collected 24h post activation **(C)**. Cells from both GLS1(+/+) and GLS(-/+) mice were treated GLS1 inhibitor JHU-212 (2.5$\mu$M) at a dose that providing only 50% inhibition of proliferation. Error bars represent +/- SEM of three mice per group. **P<0.01; ***P<0.001, ****P<0.0001 (Student's t-test).

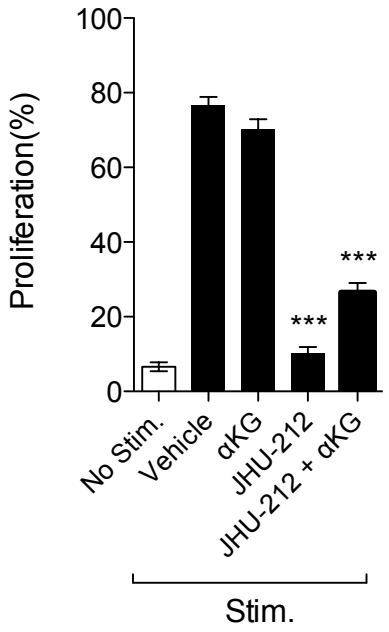


**Figure B.5:** *α-ketoglutarate (αKG) does not fully rescue proliferation in JHU-212 treated lymphocytes*
CD3+ lymphocytes were isolated from the spleens of C57BL/6 mice and treated with glutaminse (GLS1) inhibitor, JHU-212 (5$\mu$M) or JHU-212 and cell permeating $\alpha$-ketoglutarate (from BSi). CD3+ cells were activated using platebound anti-CD3/anti-CD28 (Stim.). Proliferation was assessed by CFSE dilution 72h post activation. Cells were treated with DMSO for vehicle control. Error bars represent +/- SEM of three replicates per group. ***P<0.001, (One-Way ANOVA).

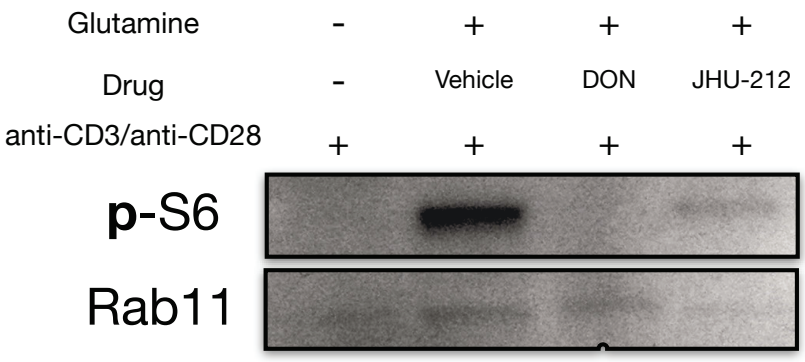


**Figure B.6:** *DON and JHU-212 treatment inhibits S6 phosphorylation after stimulation of primary T-cells*
CD3+ lymphocytes were isolated from the spleens of C57BL/6 mice and treated with glutaminse (GLS1) inhibitor, JHU-212 or DON. T-cells were activated using plate bound anti-CD3/anti-CD28. Lysates were probed for S6 phosphorylation using western blot with Rab11 serving as a loading control.

# APPENDIX C

# APPENDIX - CHAPTER 4

**Contents**



**Figures**



## C.1 Supplementary Methods

### C.1.1 Drugs

GYKI-53655 (Sigma) was solubilized in DMSO or 10% DMSO in 0.1M HCl to obtain a 26mM or 100mM stock. For in vivo experiments, stock solutions were further diluted in sterile PBS or complete media.

### C.1.2 Calcium Flux Analysis for GYKI-52466

CD3+ lymphocytes were purified from freshly isolated CD-1 female mouse spleens by column purification according to the manufacturer's protocol (Miltenyi Biotec). Lymphocytes were then resuspended in calcium-free buffer (PBS w/ 25mM HEPES, 0.1% glucose, 1mM sodium pyruvate, and 0.5% BSA) and counted using a hemocytometer. Stock concentrations (10x) of calcium-sensing dyes Fluo-4 AM and FuraRed AM (Invitrogen) were prepared in DMSO with 0.2% pluronic acid (Sigma) and 25mM probenecid (Invitrogen). Cell density was readjusted to $10^6$ cells/mL in calcium-free buffer and cells were stained with working concentrations of calcium sensing dyes (Fluo-4 AM - 1$\mu$M and Fura Red AM - 2$\mu$M with 0.02% pulronic acid, 2.5mM probenecid, final) for 45 minutes at 37℃. Lymphocytes were then washed with calcium-free buffer to remove excess dyes and resuspended at an optimal working density. An aliquot of Fluo-4/Fura Red-labeled lymphocytes were either mock-labeled (DMSO) or labeled with 1$\mu$M Calcein Violet AM (Invitrogen), a viability dye, for 30 minutes at 37℃. Cells were then washed with calcium free buffer to remove excess dye. Mock-labeled lymphocytes were treated with drug vehicle (10% DMSO in 0.1M HCl) while Calcein-labeled lymphocytes were treated with GYKI-52466 (Sigma) at 150$\mu$M. Mock and GYKI-52466-treated lymphocytes were then incubated at 37℃ for 45-60 minutes before being washed and resuspended in calcium-free buffer. Mock and GYKI-52466 cells were then incubated with biotin-conjugated anti-CD3 (5$\mu$g/mL) and anti-CD28 (1-2.5$\mu$g/mL) antibodies (eBiosciences) for 30 minutes at 4℃ and subsequently washed in calcium-free buffer and placed on ice. Mock (unlabeled) and GYKI-52466 (labeled) treated lymphocytes were mixed at a 1:1 ratio in a separate round bottom test tube and the volume raised to 1mL with calcium free buffer.

The mixed samples were then run on a BD FACS Canto II set to record on FITC, PerCP-Cy5.5, and Pacific Blue channels. After 60 seconds into acquistion to establish a baseline, the samples were treated with streptavidin (10$\mu$g/mL, Invitrogen) to crosslink CD3 and CD28 and acquisition resumed for 120 seconds. At 180 seconds after start of initial acquisition, $CaCl_2$ was added to a final concentration of approximately 1mM and data acquisition was resumed for another 5 minutes before being terminated. Flow cytometry data were viewed using FlowJo(Tree Star Inc) and exported into Excel (Microsoft) for graphing.

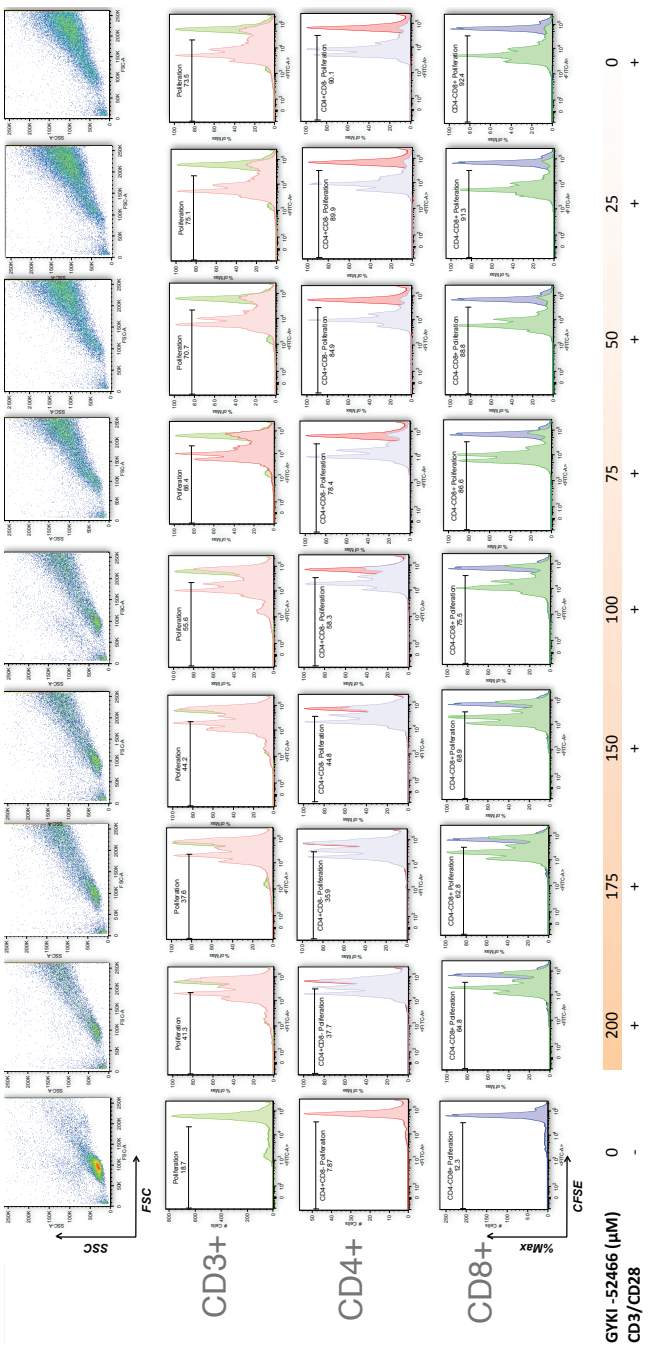


**Figure C.1:** *Effect of GYKI-52466 on lymphocyte proliferation.*
Splenocytes from CD-1 mice were harvested and activated using plate bound anti-CD3/anti-CD28. GYKI-52466 was serially diluted from 200$\mu$M - 0$\mu$M. Cells were grown in gluamte containing DMEM media with 10% Regular FBS, sodium pyurvate, B-ME, NEAAs, gentamycin, pen/strep, and IL-2. Splenocytes were stained for CD3 (PE), CD4(PerCp), and CD8 (APC). Proliferation was measured by CFSE dilution assay via flow cytometry and gated on CD4+ and CD8+ cell populations.

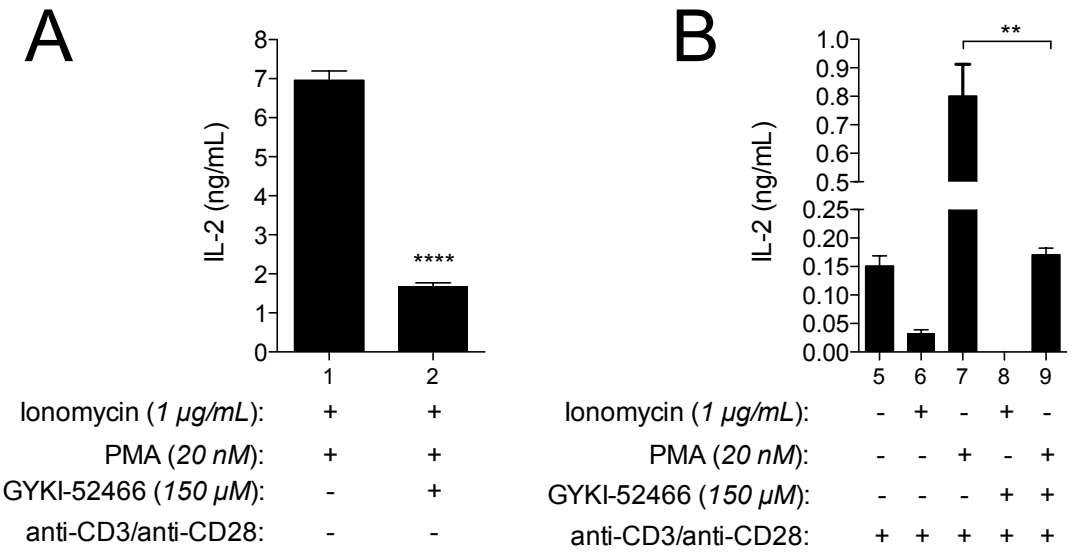


**Figure C.2:** *GYKI-52466 inhibits PMA accelerated IL-2 production in primary lymphocytes*
Purified CD3+ cells from the spleens of CD-1 mice were activated using Ionomycin/PMA or plate bound anti-CD3/anti-CD28. Cells were activated using Ionomycin/PMA, treated with GYKI, and IL-2 production measured by ELISA at 12h **(A)**. GYKI treated or untreated cells were activated using anti-CD3/anti-CD28 and were supplemented with ionomycin or PMA and IL-2 production was measured at 12h by ELISA**(B)**. Error bars represent +/- SEM of two replicates per group. **P<0.01; ****P<0.0001 (Student's t-test).

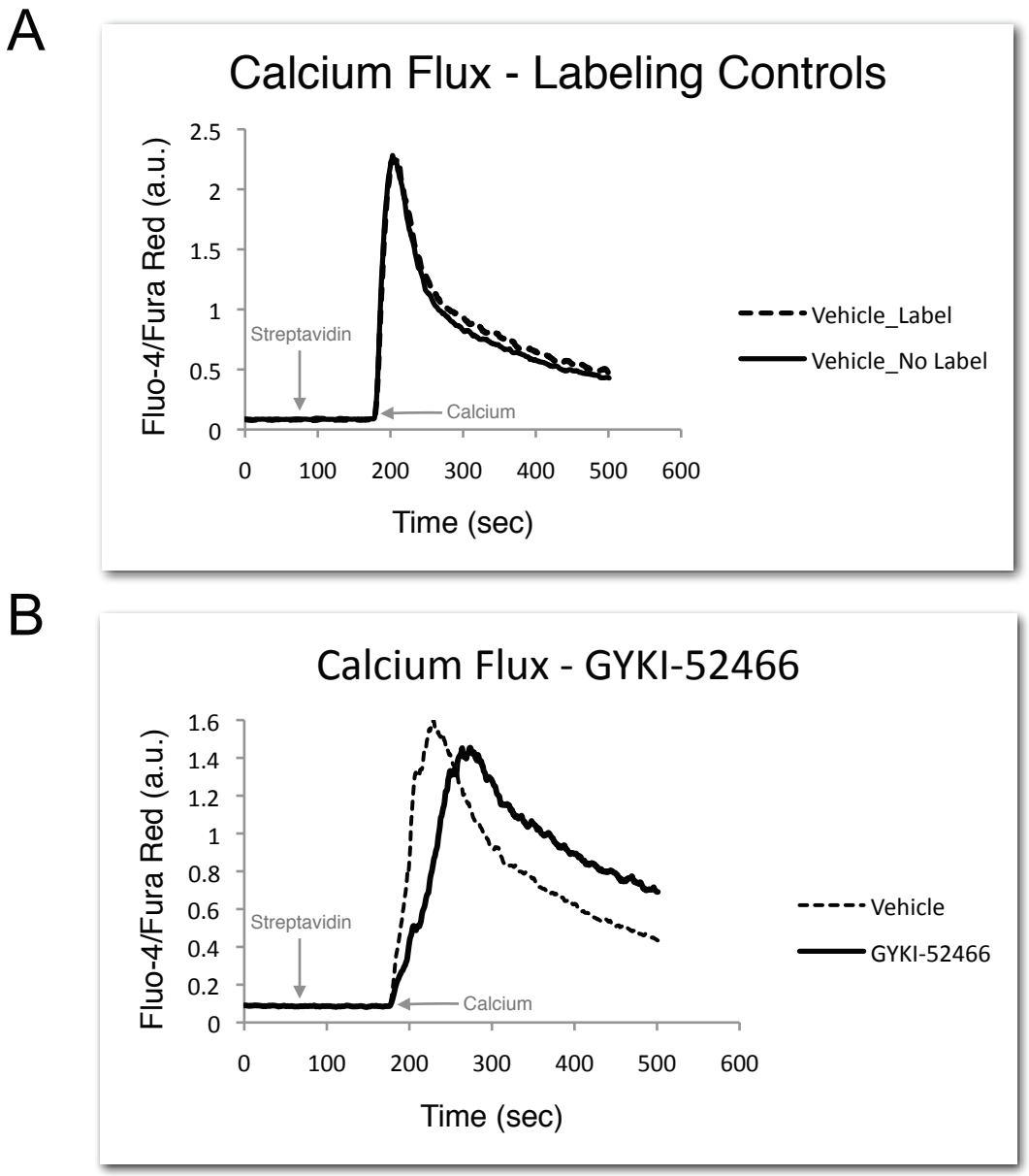


**Figure C.3:** *CD3+ T-cells treated with GYKI-52466 had a delay in external calcium flux*
Purified CD3+ lymphocytes were labeled with ratiometric calcium indicator Fluo-4/Fura-Red, pretreated with either Vehicle or GYKI-52466 (150$\mu$M). Biotin-conjugated anti-CD3/anti-CD28 was added at 60sec and $CaCl_2$ at 180sec.

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# Sivabalan Manivannan


Contact Information

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*Location of Birth:* Tamil Nadu, India
*DOB:* 09/12/1984
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Citizenship

*United States of America*

Education

**Johns Hopkins University** Baltimore, MD

**PhD**, Molecular Microbiology and Immunology December 2014

Bloomberg School of Public Health
Department of Molecular Microbiology and Immunology
Thesis: *Treatments for acute viral encephalitis.*
Adviser: Dr. Diane E. Griffin, MD, PhD
Courses in basic molecular and cell biology, immunology, virology, bacteriology, parasitology, epidemiology, biostatistics, and public health.

**ScM**, Molecular Microbiology and Immunology August 2009

Thesis: *Packaging of AID into HIV Virions and The Presence of AMPA Receptors on Lymphocytes*
Advisor: Dr. Diane E. Griffin, MD, PhD
Co-Advisor: Dr. Xiao-Fang Yu

**University of Alabama at Birmingham** Birmingham, AL

**BSc**, Physics and Biology December 2007

Courses included a strong mix of mathematics, physics, biology, chemistry, and computer science.

Publications


Murray, C. J. L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C.,. . . , **Manivannan S**, et al. (2012). *Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 380(9859), 2197–2223. doi:10.1016/S0140-6736(12)61689-4

Vos, T., Flaxman, A. D., Naghavi, M., Lozano, R., Michaud, C., Ezzati, M.,. . . , **Manivannan S**, et al. (2012). *Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 380(9859), 2163–2196. doi:10.1016/S0140-6736(12)61729-2

## Conferences and Talks

**Manivannan, S**, Slusher B, Griffin, DE (2013). *Glutamine antagonist, DON, protects mice from acute fatal encephalomyelitis by inhibiting T-cell growth and proliferation.* International Society for NeuroVirology (Washington, DC) - Poster and Talk

**Manivannan, S**, Slusher B, Griffin, DE (2013). *Glutamine antagonist, DON, protects mice form acute fatal encephalomyelitis by inhibiting T-cell growth and proliferation.* Keystone Meeting: Metabolic Control of Inflammation and Immunity (Breckenridge, CO) - Poster

**Manivannan, S**, Griffin, DE (2012). *AMPA receptor antagonists inhibit lymphocyte proliferation.* Frontiers in Basic Immunology (Washington, DC - NIH Campus) - Poster

**Manivannan, S**, Griffin, DE (2010). *AMPA receptors are present on mouse lymphocytes and AMPA receptor antagonist, GYKI-52466, inhibits lymphocyte proliferation.* American Association of Immunologists (Baltimore, MD) - Poster

## Awards

Johns Hopkins Bloomberg School of Public Health

| | |
|---|---|
| Third Place in Delta Omega Poster Competition | 2014 |
| Student commencement speaker - 2013 JHSPH graduation | 2013 |
| Recognition Award - Outstanding Service as Student Assembly President | 2013 |
| Dr. Bettylee Hampil Fellowship for Doctoral Research in Immunology | 2010 |

## Teaching

Johns Hopkins Bloomberg School of Public Health

**Public Health Biology**: *Teaching Assistant* Spring and Fall '10 '11 '12

Instructors: Dr. David Sullivan MD, Dr. George Korch, PhD
A distance learning course that provides an introduction to molecular biology and the biological basis of public health for students without a science background.
Graded exams, answered questions on the class's online Q&A system, and provided ancillary support for live talk sessions between faculty and students.

## Research

Johns Hopkins Bloomberg School of Public Health

**Treatments for acute fatal alphaviral encephalomyelitis** May 2007 to Current

Advisor: Dr. Diane Griffin, MD, PhD
Worked on two projects that involved non-canonical drug treatments to mitigate Neuroadapted Sindbis Virus induced fatal encephalomyelitis.
Worked closely with the Brain Science Institute and their drug discovery team to evaluate potential drug candidates for efficacy in our mouse model.
Determined the mechanism of action of drugs using *in-vitro* primary and transformed lymphocyte cell culture, qRT-PCR, ELISA, flow cytometry and western blots.
This research provided insights into potential treatment strategies for alphavirus induced fatal encephalomyelitis.

**Evaluating the global disease burden of Bordetella Pertussis.** May 2008 to June 2008

Investigator: Dr. Saad B. Omer, MBBS, PhD
Conducted a systematic literature review evaluating Pertussis incidence, mortality, prevalence, and vaccine coverage worldwide.
Reviewed literature and abstracted relevant data while designing, creating, and maintaining the database utilized to store information gathered by the entire team.
The Pertussis data will be incorporated into the comprehensive Global Burden of Disease report issued by a consortium based at the Johns Hopkins Bloomberg School of Public Health.

**Packaging of AID into HIV Virions** May 2008 to June 2008

Advisor: Dr. Xiofang Yu, MD, ScD
Studied the viral determinants required for the packaging of Activation induced Cytidine Deaminase (AID), an ancestral homolog of the APOBEC family, into HIV virions using in-vitro cell culture and western blots.

University of Alabama

**Microbial community structure of undersea tunicates** Jan. 2007 to Apr. 2007

Advisor: Dr. Julie Olson, PhD
Utilized common culturing and molecular techniques (mainly T-RFLP) to obtain a fingerprint of the microbial flora inhabiting tunicates harvested in different areas off the coast of Panama.

## Grants Written and Funded

Johns Hopkins Bloomberg School of Public Health

**JHMRI Pilot Research Grant** 2012 - 2013

*Glutaminase Inhibitors and AMPA Receptor Antagonists as potential Novel Therapeutics for Cerebral Malaria*
Brain Science Institute, Johns Hopkins School of Medicine
Funding: $75,000
PI: Diane Griffin, MD, PhD
Summary: Explored the use of glutaminase inhibtors for the treatment of cerebral malaria.

**Brain Science Institute Translational Research Grant** 2012 - 2013

*Glutaminase Inhibition as a Novel Immunomodulatory Treatment for inflammatory Neurologic Diseases*
Johns Hopkins Malaria Research Institute
Funding: $40,000
PI: Diane Griffin, MD, PhD
Summary: Studied the effects of glutaminase inhibitors on acute viral encephalitis.

## Leadership Experience

Johns Hopkins Bloomberg School of Public Health

**Student Assembly**: *President '13, President-Elect '12* 2010 - 2014

Student Assembly is the student government organization for JHSPH.
Guided the allocation of a total budget of over $100,000 with the Treasurer.
Managed a total of 35 officers including 12 Vice Presidents responsible for providing the financial and resource infrastructure for student groups.
Organized, curated, and ran complex school-wide and interschool events.
Introduced and integrated online project management system, Basecamp, to the Student Assembly which helped preserve institutional knowledge.
Student convocation speaker at 2013 JHSPH graduation ceremony.

## Laboratory Skills

*Experimental Skills*

Experimental design, execution, and analysis of wet lab experiments
Flow cytometry
Mice handling and tissue harvesting
Western Blot
RNA isolation from tissues and cell culture
qRT-PCR and PCR
Drug dose response studies *in-vivo* and *in-vitro*
Dissolving compounds using different excipients.
Immunohistochemistry
Transfections
Primary and transformed cell culture
*In vivo* and *in vitro* proliferation assays
Miltenyi MACS Cell Seperation
ELISA (cytokines, IgG, IgM)
Plaque assay
Custom media formulation

## Technical Skills

*Programming and Computer Skills*

Interpreted Languages: Python, Perl, R
Compiled Languages: C, Go, FORTRAN90, C++
Web Design: HTML5, Javascript (jQuery, AngularJS), CSS
Web Framework: Django 1.6, a Python based web framework
Proficient in shell scripting (BASH) including the use of command line parsing tools (*sed*, *awk*, *grep*, *etc.*) to clean and organize data.
Regular expressions
Databases: SQL, PostgresSQL, MySQL, MongoDB
Familiar with basic algorithms and data structures
Version Control: Git
Familiar with Linux containers: Docker.io
Operating Systems: Ubuntu Linux, BSD Unix (MacOS)

*Statistical / Data Analysis Software*

Prism, a basic statistical analysis and graphing software for lab scientists.
NumPy, a Python library for scientific computation.
Pandas, a Python library for data analysis

IPython Notebook, a web based computational data analysis environment
R, a statistical programming language and data visualization package

*Typesetting and Productivity Software*

Familiar and comfortable producing documents in LaTeX.
Microsoft Office Suite (Word, Excel, PowerPoint)
Apple Keynote Presentation Software